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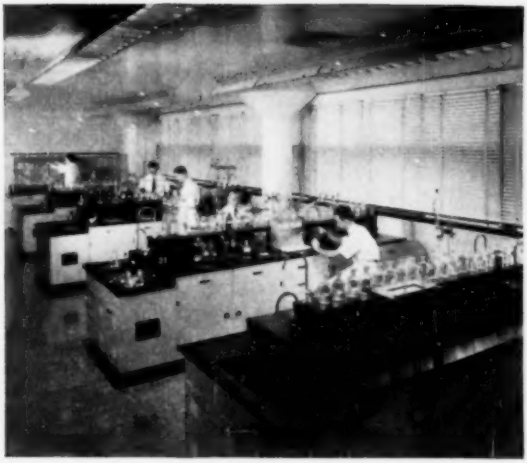

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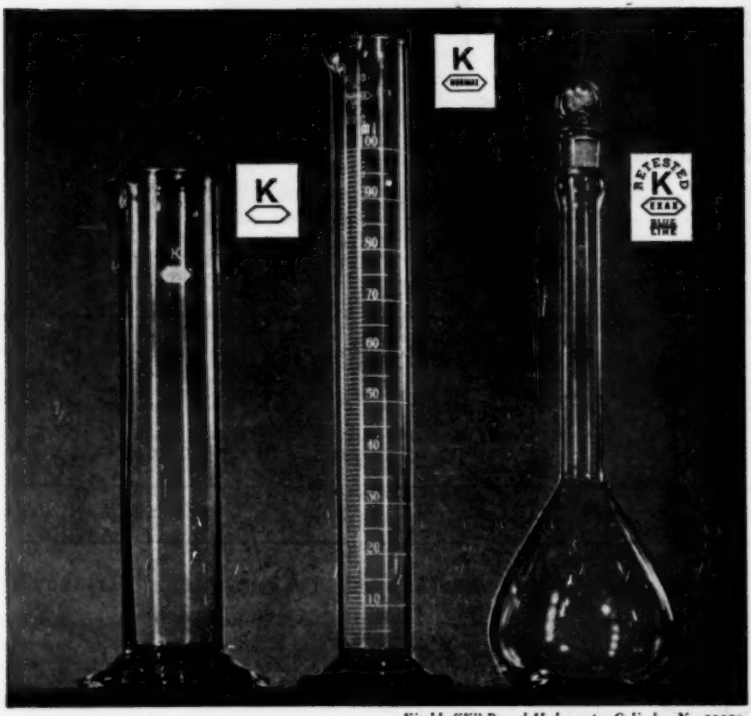

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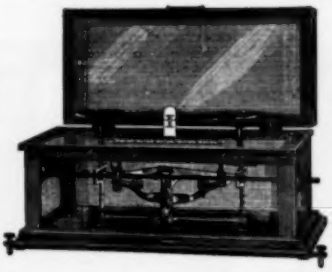

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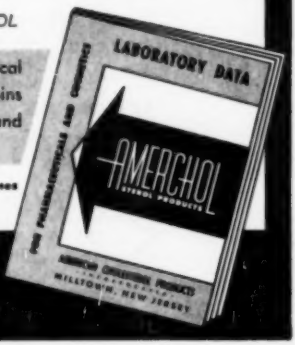

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Evaluation of Hydrophilic Properties of Bulk Laxatives Including the New Agent, Sodium Carboxymethylcellulose*

By RUDOLPH H. BLYTHE, JOHN J. GULESICH, and HARLAN L. TUTHILL

This study was undertaken to find a laxative agent superior to those now in use. The hydrophilic properties of standard bulk laxatives are compared with two new synthetic agents. New and modified methods for the *in vitro* evaluation of such agents are described.

THE NATURAL gums have recognized faults when used as bulk laxatives. Clinically, they may cause antigenic reactions (1, 2) and lead to obstructions (1, 2) or a feeling of flatulence (3). Pharmaceutically, different batches show variation in gel strength and cleanliness. It was evident that a synthetic hydrophilic substance would eliminate the pharmaceutical variables and might be superior clinically. The cellulose derivatives, methylcellulose (MC)[1] (4) and sodium carboxymethylcellulose (CMC)[2] (5, 6), are strongly hydrophilic, resistant to digestion and nontoxic. They were, therefore, compared with various vegetable colloids.

The *in vitro* tests (1, 3) heretofore used to evaluate laxatives are only distantly related to conditions in the gastrointestinal tract. Gray and Tainter (1) supplemented their laboratory tests with clinical data on wet and dry stool weights. In the present study, however, work with the stool weight method, although attempted, was discontinued when it was found that statistically significant data could not be obtained under conditions practical for the investigation. It appeared that it would be necessary to place a very large number of institutionalized people on a standard diet for a considerable period of time. Since this would create an artificial condition, it seemed preferable to develop a physicochemical measurement that would bear a similarity to the physiological process and eliminate biological variabilities.

* Received August 9, 1948, from Smith, Kline & French Laboratories, Philadelphia, Pa.

Presented to the Subsection on Pharmacy of the American Association for the Advancement of Science at its Annual Meeting, Chicago, Ill., Dec. 27, 1947.

The authors wish to thank Edmund W. Wydrzynski and Catherine F. DeFonso for their technical assistance in this investigation.

[1] Methylcellulose High Viscosity, Dow Chemical Co.

[2] Sodium Carboxymethylcellulose High Viscosity, Hercules Powder Co.

Three properties are significant in the *in vitro* evaluation of bulk laxatives: (*a*) the volume of water absorbed in various media (water and artificial gastric and intestinal juices), (*b*) the viscosity and texture of the gel formed, and (*c*) the ability of the gel to retain water.

To avoid causing gastric discomfort, the agent should swell in the alkaline intestinal tract but not in the acid gastric juice. The physical characteristics of the gel determine its ability to mix with the intestinal contents; a high degree of miscibility may be the primary factor in avoiding the formation of gummy impactions in the gut. In order to keep the intestinal contents soft throughout the tract, the gel must be able to retain its water against pull of osmotic and other water-absorbing forces within the intestinal wall.

EXPERIMENTAL

Method I. Gel Volume and Liquid Absorption.—Add 5 Gm. of the hydrophilic material to 500 ml. of liquid (water or artificial gastric juice or artificial intestinal juice) in a 500-ml. graduated cylinder. Agitate contents gently by inverting every fifteen minutes for two hours; then let stand undisturbed for eighteen hours. Note the volume to which the gel has swollen (Gel Volume) and pour the material onto a moistened, fine glass wool filter ($3^1/_2$ inches square and $^1/_4$ inch thick, weighing 2 Gm.) held in a 6-inch glass funnel. Allow the liquid to drain for five minutes into a second 500-ml. graduate. Calculate by difference the volume of liquid retained by the gel (Liquid Absorption). As a variation of the above procedure, a single layer of unwashed absorbent cheesecloth (Grade 80) is used as the filter medium in place of the glass wool.

FORMULA FOR ARTIFICIAL GASTRIC JUICE

Hydrochloric Acid	0.60%[1]
Pepsin	0.05%

FORMULA FOR ARTIFICIAL INTESTINAL JUICE

Sodium Bicarbonate	1.50%
Bile Salts (Bacto)	1.00%
Pancreatin	0.28%

Method II. Gel Volume with Increased Agitation.—Proceed as in Method I but omit manual inversion and place test mixture in a quart bottle and rotate end over end mechanically at 36 r. p. m. for four hours, pour contents into 500-ml. graduate and allow to stand eighteen hours before reading.

Method III. Water Retention against Osmotic Pull.—The apparatus, as shown in Figs. 1 and 2, is of our own design. The method is based upon the theory that the laxative efficacy of a hydrophilic colloid is proportional to its ability to hold water against absorption from the intestinal tract into the blood stream. For comparative purposes, such efficacy can be quantitatively determined by measuring the ability of a sol or gel to hold water against the osmotic pull of a hypertonic solution when the two media are separated by a semipermeable membrane. As standard conditions of test, we selected a 170-mm. column of a 30% aqueous solution of "Carbowax" 4000W[4] as the hypertonic solution and separated it from a 2% concentration of the hydrophilic colloid being tested by a 600A PT cellophane membrane having an effective diameter of 50 mm.

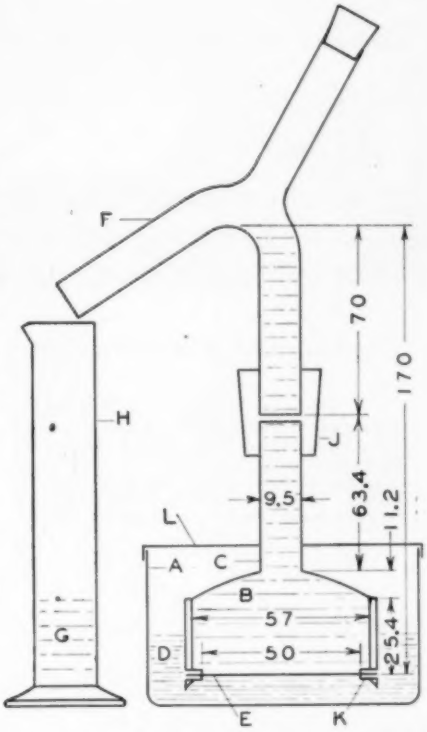

Fig. 1.—Apparatus for determination of water retention against osmotic pull (dimensions in mm.).

A, 90-mm. crystallizing dish; *B*, hypertonic solution; *C*, brass osmometer; *D*, test mixture, 2%; *E*, 600A. PT cellophane (Du Pont) 58 mm. diameter; *F*, 10-mm "Y" tube. Length of arm, 70 mm.; *G*, overflow from osmometer; *H*, 25-ml. graduate; *J*, rubber stopper connection; *K*, rubber washers (Ball No. 10, top seal jar rubbers) held in place with a metal ring and clamps; *L*, "Parafilm" membrane (Menasha Prod. Co., Menasha, Wis.).

[1] Based on Bodansky, Meyer, "Introduction to Physiological Chemistry," ed. 4, John Wiley and Sons, New York, 1938, p. 169. This reference indicates that the concentration of hydrochloric acid as it is secreted is approximately 0.17 *N* or about 0.62%, and the pH is approximately 0.88; the pH of the formula above is 1.0. The pH is an arbitrary one used for convenience, since test conditions prohibit making allowances for differences that occur among persons with varying stomach content or the continuing supply of fresh gastric juice.

[4] Brand of polyethylene glycol obtained from Carbide & Carbon Chemicals Corp.

The test is carried out as follows: Prepare the test mixture by adding 2 Gm. of the hydrophilic material to 98 Gm. of artificial intestinal juice and mix occasionally over a two-hour period. Allow the mixture to stand for eighteen hours so that the hydrophilic substance may become hydrated. Pour this mixture into the glass crystallizing dish (*A*) (see Fig. 1). Pour the hypertonic solution (*B*) into the osmometer (*C*) until it rises in the neck. Then place the osmometer in the test mixture (*D*). Exercise care to avoid trapping air bubbles under the cellophane membrane (*E*). Now fill the osmometer with the "hypertonic solution" until it starts to drop out at the lower, open end of the "Y" tube (*F*). Place a "Parafilm" or "Pliofilm" membrane over the crystallizing dish to reduce evaporation of the hydrophilic gel. Collect the overflow (*G*) (mixture of the "hypertonic solution" and liquid pulled from the hydrophilic gel through the membrane) in 25-ml. graduated cylinders (*H*). Record the volume of liquid obtained over a period of forty-eight hours. Figure 2 is a photograph of the above described setup.

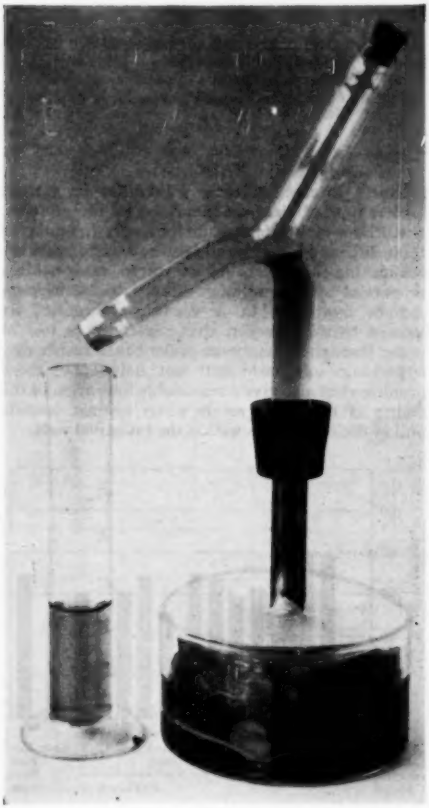

Fig. 2.—Apparatus for determination of water retention against osmotic pull.

DISCUSSION

Effect of the Media on Gel Volume (Table I, Figs. 3 and 4).—The influence of the medium on gel volume is shown in Table I and Fig. 3 (illustrating results with Method I) and Fig. 4 (Method II). All the substances show marked differences in behavior. As has already been observed (1) the karaya and psyllium type substances swell much better in water than in artificial physiological fluids. In the simulated gastric juice they attain, roughly, half the volume obtained in water, and in artificial intestinal juice they swell more than in the acidic medium but less than in water. This is true whether Method I, involving only eight inversions at fifteen-minute intervals, or Method II, continuous mild agitation over a four-hour period, is used.

The cellulose derivatives, on the other hand, dissolve equally well in water or in artificial intestinal fluid. They show an important difference in behavior, however, in the simulated gastric juice. When either Method I or Method II is used, sodium carboxymethylcellulose is converted to the free acid which is insoluble; but methylcellulose becomes partly swollen and floats on the surface of the "gastric" medium when Method I is used, and with Method II it goes into complete solution in all three media.

The fact that sodium carboxymethylcellulose does not swell in artificial gastric juice, even when agitated to a degree which reasonably approximates conditions *in vivo*, suggests a clinical advantage. Unlike many bulk laxatives, it should not produce an uncomfortable feeling of fullness. Its insolubility in the artificial gastric juice does not interfere with its efficacy since, upon removal from the "gastric" to the "intestinal" medium, sodium carboxymethylcellulose is reconverted to the soluble sodium salt and readily dissolves.

Viscosity and Texture of the Gel (Table I, Fig. 3).—The cellulose derivatives form a clear viscous solution. Karaya products swell in the various liquids to form gels which retain their granular texture. Psyllium products may be classed between these two extremes. Whole psyllium seed forms gelatinous masses with the inner part of the seed retaining much of its form. The hemicellulose materials derived from psyllium give viscous, pourable, opaque gels with widely varying amounts of insoluble seed material remaining, depending on the brand of product. The character of the gel formed by each test material is shown in Table I.

These characteristics may have important clinical significance. In the first place, a solution should be fully miscible with the intestinal contents and not remain as the gelatinous masses mentioned by Ivy and Isaacs in their study of a karaya product (3). This expectation was substantiated on examination of stools of mice fed sodium carboxymethylcellulose (7). Secondly, the unique ability of the cellulose derivatives to pass through the very small openings of the cheesecloth (Fig. 3) suggests that they are less likely than the gums to form an impaction in a partially obstructed intestine.

Retention of Water (Fig. 5).—Some indication of hydrophilic capacity of certain test substances is obtained with Method I. However, the physical differences between the synthetics and the natural products limit the significance of this test when it is

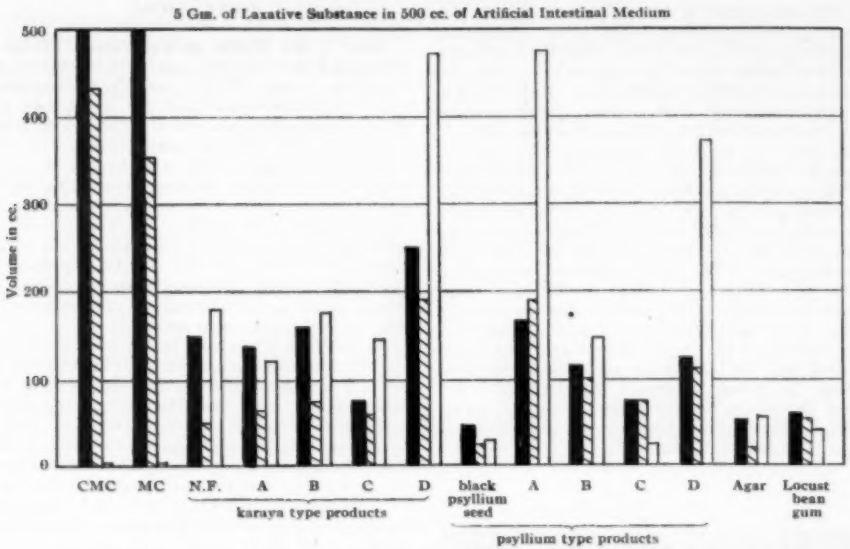

Fig. 3.—Gel volume and liquid absorption (method I). 5 Gm. of laxative substance in 500 cc. of artificial intestinal medium. Key: black, gel volume; shaded, liquid retained on glass wool; white, liquid retained on cheesecloth. Substances indicated above as karaya type products and psyllium type products A to D, inclusive, represent leading commercial products available under various trade names.

applied to dissimilar substances. Method III, devised to minimize the influence of these conditioning factors and to approximate *in vivo* conditions, gives results which seem more significant. As shown in Fig. 5, the cellulose derivatives are clearly superior in hydrophilic properties; the relative efficacy of the various karaya and psyllium preparations differs widely with the source and brand of the product tested.

NEW "HYDROPHILIC" TEST

The factors which limit the value of Methods I and II are viscosity and nature of the gelatinous mixtures formed, differing solubilities in various media, type of filter used, and degree of agitation. A search was therefore made for a way to minimize these differences. Ivy and Isaacs (3) were probably attempting this when they compared water-holding properties by measuring resistance to drying over sodium hydroxide in a vacuum desiccator. It seemed to us, however, that resistance to loss of water through a membrane under the influence of a hypertonic solution might not only solve these problems but also give a reasonable indication of the ability of the gel to retain water against osmotic pull of the body fluids within the intestinal wall.

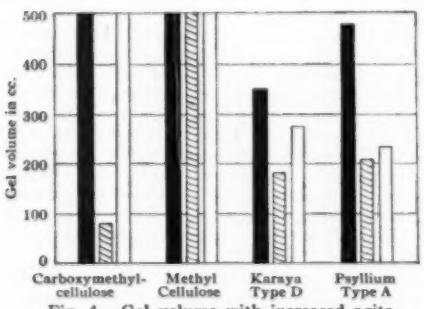

Fig. 4.—Gel volume with increased agitation (method II). Key: black, water; shaded, artificial gastric juice; white, artificial intestinal juice.

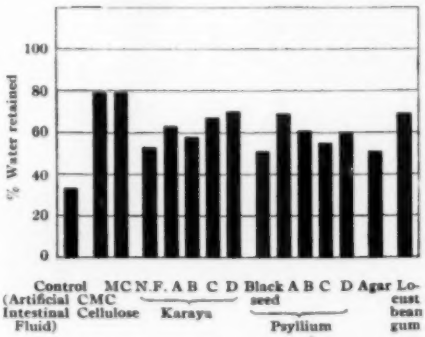

Fig. 5.—Water-holding capacity—against osmotic pull (method III). Forty-eight hour reading.

TABLE I.—GEL VOLUME AND LIQUID ABSORPTION

	Water			Artificial Gastric Juice		Artificial Intestinal Juice		
	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Consistency of Material on Filter	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Gel Volume, ml.	Liquid Retained on Glass Wool, ml.	Liquid Retained on Cheese Cloth, ml.
I. Cellulose Type								
CMC	500	470	Viscous sol	[a]	40	500	430	0
MC	[b, c]	[c]	[c]	[b, c]	[c]	500	355	0
II. Karaya Type								
Karaya, N. F.	205	80	Stiff and lumpy	95	35	150	50	180
A	195	90	Stiff, granular gel	95	40	135	65	120
B	270	140	Stiff, granular gel	115	80	160	75	175
C	180	120	Stiff, granular gel	60	40	75	60	145
D	350	230	Viscous, granular gel	165	125	250	190	470[d]
III. Psyllium Type								
Psyllium Seed Black	55	25	Stiff gel	30	10	45	25	30
A	370	310	Slightly viscous	180	175	165	190	475[d]
B	125	110	Slightly viscous	100	95	115	100	145
C	90	65	Slightly viscous	80		75	75	25
D	175	165	Watery	105	90	125	110	370[d]
IV. Agar	60	45	Soft gel	40	20	50	20	55
V. Locust Bean Gum	133	155	Watery	75	80	60	55	40

[a] Carboxymethylcellulose is converted to the free acid, which is insoluble.
[b] Material floats on surface of liquid in a partially swollen condition.
[c] Does not go completely into solution.
[d] Gum forms film on cheesecloth that prevents unbound liquid from draining.

Method III was, therefore, devised. As shown in Fig. 1, the gel or sol was separated by a cellophane membrane from a hypertonic solution, and the loss of liquid from the gel or sol was measured by the overflow from the osmometer. The remarkably superior water-holding capacity of the synthetic cellulose derivatives is evident from the data in Fig. 5. For comparative purposes, observations were made at forty-eight-hour intervals since, as shown in Fig. 6, the more effective substances approach an equilibrium at that time. Thus longer periods of observation (e.g., one-hundred-twenty hours) were unnecessary.

The use of a polyethylene glycol as a hypertonic agent deserves some comment. This substance was chosen because its large molecular size minimizes its passage through the cellophane membrane. Tests with the 30% solution indicated that after seventy-two hours not more than 1.1% of the polyethylene glycol has passed through the membrane into distilled water on the other side. This concentration makes significant readings obtainable within a relatively short time. The tonicity of 30% solution was calculated as 5.3 on the basis of a freezing-point depression of 2.96° C.

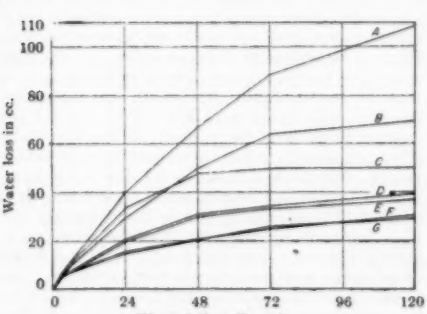

Fig. 6.—Water-holding capacity—against osmotic pull. Water loss rate. Key to curves: *A*, artificial intestinal juice[1] (control); *B*, psyllium seed black; *C*, karaya; *D*, psyllium type A; *E*, karaya type D; *F*, MC; *G*, CMC.

[1] An additional 50 ml. of medium was added to keep membrane submerged.

SUMMARY

1. New and improved *in vitro* tests are described for the evaluation of hydrophilic laxatives. These tests measure swelling and water retention in media reasonably similar to those of the stomach and intestinal tract. *In vivo* conditions are further simulated by a novel use of polyethylene glycol as an osmotic agent.

2. Commercial preparations of karaya and psyllium and the synthetic agents methylcellulose (MC) and sodium carboxymethylcellulose (CMC) are compared by means of these tests.

3. One significant difference between the two synthetics was observed: When agitated in simulated gastric juice, MC goes into solution slowly but CMC remains essentially insoluble.

4. The physicochemical properties of the cellulose derivatives give them two advantages over the natural gums: greater hydrophilic capacity and the formation of viscous, homogeneous solutions. The former makes a more efficient bulk laxative. The latter permits uniform distri-

bution through the intestinal contents and should eliminate any tendency to produce intestinal blockage.

5. Because of the general superiority of the synthetic substances over the natural products and specifically because CMC is insoluble in simulated gastric juice, it is concluded that for use as a bulk laxative CMC is the most promising substance of all those tested.

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Effect of the Rate of Injection of Digitalis Upon Its Lethal Dose in Guinea Pigs*†

By TOM S. MIYA and HARALD G. O. HOLCK‡

Eighty-four male guinea pigs were divided into seven groups of twelve each and anesthetized with urethane. Each group was tested with varying concentrations of U. S. P. Reference Standard Digitalis (1942) Tincture, using normal saline solution as the diluent. The effects of varying the concentration of digitalis tincture are reported. The results obtained are compared with those of other investigators and recorded in this report.

ALTHOUGH the specifications for the cat in the assay of digitalis have been relaxed by allowing a wider weight range in the latest revision of the U. S. P. (1) it has become increasingly difficult to obtain cats falling within these limits. Therefore, interest has recently been revived in the possible use of the guinea-pig method introduced by Knaffl-Lenz (2) as a substitute. Several recent studies have been made dealing with the use of this animal (3, 4) to which we shall refer in the discussion.

The purpose of this study was to ascertain the effects of varying the concentration of the digitalis tincture and to compare the results with those of other investigators and also with those obtained in the cat studies of Holck, Kimura, and Bartels (5).

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† This project was aided by a grant to the Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, from Ciba Pharmaceutical Products, Inc.
‡ We are indebted to Professor E. Fullerton Cook for supplies of U. S. P. Reference Standard Digitalis Powder (1942) and to Professor C. T. Blunn for aid in the mathematical analysis.

EXPERIMENTAL

Eighty-four male guinea pigs were used in our main study on the effect of varying concentrations and fourteen in the second study on the effect of alcohol on the lethal dose. All the animals were obtained from the same source (Scottdale, Pennsylvania) so as to prevent as much as possible any variations in lethal dose which might occur due to difference in strain or sex, as well as any extraneous and unknown causes as reported in the use of cats by Bliss and Hanson (6). Seven different dilutions of the digitalis tincture, prepared according to the U. S. P. XIII and diluted with physiological saline solution were injected. From the lowest concentration, these increased in strength by 41.42%, giving an increase of 100% for each alternate concentration. To avoid complications which might occur if the animals were tested over a long period of time, seven animals forming a group were usually injected within a period of one week and, in no case, more than two weeks, with a concentration of 1.60, 2.26, 3.20, 4.53, 6.40, 9.05, and 12.80% of the tincture of digitalis, respectively. The order of testing the dilutions was varied with the aid of 7 x 7 Latin squares as given in the tables of Fisher and Yates (7). Because there have been conflicting reports of the effect of the weight of the animals on the lethal dose (2, 3, 8) it was decided to use a narrow range of weights (480–600 Gm.), and to distribute the animals in such a manner that the average weight in each group was nearly the same. A 25% solution of urethane was used to produce anesthesia. An initial dose of 1.0 Gm./Kg., was administered intraperitoneally and supplemented with small doses as needed. All the tests were carried out between October 17, 1947, and January 16, 1948, a period of three months.

The general procedure was that of the U. S. P. XIII (1) except that injections were made into the jugular instead of the femoral vein. Although the Pharmacopœial fasting range for cats is sixteen to

twenty-eight hours, our variations were kept within twenty to twenty-eight hours. The tests were conducted with tinctures not older than seventeen days, whereas the Pharmacopœial tolerance is thirty days. Determination of the end point was difficult because irregular and labored respiration set in before cessation of the heart beat and because the beats became sporadic before their final stoppage. The injection which caused the final stoppage of the heart was determined by auscultation and a subsequent inspection of the heart. All animals were examined and results with any guinea pigs showing gross pathological changes of the lungs were disregarded. This was done because of the finding of Holck, Smith, and Schuler (9) that cats with lung abnormalities died with a lower dose of digitalis.

RESULTS AND DISCUSSION

The main experimental data are summarized in Tables I and II.

Hildebrandt (11) and Marsh and Farah (12) found that with Strophanthin-G and various digitalis glucosides, the lethal dose was high in cats with high rates of injection and low with slow rates of injection using diallylbarbituric acid (Dial) for anesthesia, which is in general the reverse of the results obtained by Østling (10) and in this study upon guinea pigs using urethane anesthesia.

In comparing the reports of lethal doses of digitalis on guinea pigs by other workers we found that in no case did the doses agree satisfactorily with our results. Allmark and Bachinski (3) reported the following: 132.5, 125.0, 139.0, 127.9, and 130.0 mg./Kg. of digitalis per guinea pig with concentrations 2.50, 3.33, 5.00, 6.67, and 10.00%, respectively, using urethane anesthesia. There was no significant difference in mean lethal doses with the five concentrations. In the comprehensive investigation of Braun and Siegfried (13) it was noted that the lethal dose was high for light, and low for heavy animals. Much greater uniformity of response was obtained after applying a size factor. Because the body

TABLE I.—SUMMARY OF RESULTS FROM TESTS WITH U. S. P. REFERENCE STANDARD DIGITALIS (1942) IN 84 GUINEA PIGS UNDER URETHANE ANESTHESIA[a]

Concentration of Tincture, %	Mean Weight, Gm.	Mean No. of Injections	Mean Lethal Dose, Mg./Kg. Arithmetic[b]	Mean Lethal Dose, Mg./Kg. Geometric[c]
A. 1.60	513	113	180.7 ± 8.2 (4.5%)	178.7 ± 7.8 (4.4%)
B. 2.26	524	77	174.9 ± 8.7 (4.9%)	172.6 ± 8.5 (4.9%)
C. 3.20	527	54	173.8 ± 6.6 (3.8%)	172.7 ± 6.5 (3.8%)
D. 4.53	520	38	171.0 ± 6.6 (3.9%)	169.6 ± 6.7 (3.9%)
E. 6.40	525	25	157.9 ± 7.7 (4.9%)	155.9 ± 7.5 (4.9%)
F. 9.05	521	16	144.8 ± 6.7 (4.6%)	143.2 ± 6.2 (4.3%)
G. 12.80	520	11	146.1 ± 6.4 (4.3%)	144.6 ± 6.3 (4.3%)

[a] Twelve guinea pigs constitute each subgroup.
[b] The standard error in terms of percentage of the average is given in parentheses.
[c] The standard error for the geometric mean was calculated from the formula $2.3026\ (LD_{50})S_y$. Sy is the standard error of the mean log-dose.

The average lethal dose increased in general as the concentration of the tincture decreased with the exception of the highest concentration (12.80%) which gave a slightly higher lethal dose than the next lowest concentrations.

In comparing the results with those of the cat studies of Holck, *et al.* (5), we have omitted the highest concentration used in our study in the final analysis of comparison as this concentration was not employed in the cat studies.

A curved line with a negative slope (Fig. 1) was found to best fit the plotted points (log concentrations against geometric log doses) as contrasted to a straight line with a positive slope obtained by Holck, *et al.* (5), in cats with the identical first five concentrations also using urethane anesthesia. A curve similar to ours with guinea pigs, likewise under urethane anesthesia, was obtained by Østling (10) who observed that the lethal dose of Strophanthin-G in guinea pigs rose sharply up to 150 minutes of injection time and then became constant. In our case the leveling of the curve occurred between 25 and 38 injections (125 and 190 minutes, respectively). The primary comparisons are concerned with studies in which urethane anesthesia was employed, because it has been repeatedly shown that the choice of anesthetic makes a difference in the fatal dose of digitalis (5, 9).

TABLE II.—MEAN LOG-DOSES (GEOMETRIC) FOR DATA GIVEN IN TABLE I AND PLOTTED IN FIGURE 1

Log-Concentration Digitalis	Mean Log-Doses per Guinea Pig in Mg./Kg. for the Six Lowest Concentrations
0.204	0.25223
0.355	0.23711
0.505	0.23726
0.656	0.22933
0.806	0.19276
0.957	0.15609

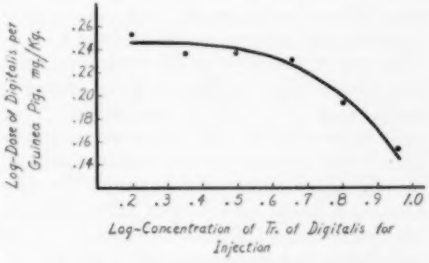

Fig. 1.—A plot of the mean log-doses for data presented in Table II.

weights in our study were well distributed within a narrow range we have used the conventional method of calculating the lethal dose. It is of some interest to note that with each of three digitalis powders studied by Braun and Siegfried the lethal dose of digitalis was lower with urethane than with ether anesthesia, whereas in the cat it has been repeatedly reported that the fatal dose of digitalis with urethane anesthesia was significantly higher than with ether anesthesia (5, 9, 14). These authors also reported that source, sex, and fasting had no appreciable effect upon digitalis toxicity in the guinea pig. It is also worthy of note that the three collaborators under the supervision of the Committee of Physiological Testing of the Scientific Section of the A. PH. A., using controlled conditions and employing local anesthesia at the site of injection, reported varying lethal doses of 125.1, 182.2, and 135.5 mg./Kg. of digitalis, respectively, with a concentration of 1:15 (4).

The mean lethal doses for the first 6 and second 6 animals used in each group were calculated. The average doses increased for groups A, B, C, and G for the second 6 animals and decreased for D, E, and F, thus no seasonal variation was evident during the testing period.

Determination of whether the higher alcoholic content of the stronger concentrations was responsible for the low mean lethal doses obtained in these groups (E, F, G) was made by testing fourteen animals (seven in each group) in a subsequent study with a concentration of 4.53%, to which enough alcohol was added to make the concentration of alcohol equal that of the 12.80% tincture. This addition of alcohol did not cause any significant added toxicity (148.8 mg./Kg. for the regular dilution and 144.8 mg./Kg. for the tincture with added alcohol). However, in this second test the fatal doses were significantly lower than those obtained in the main investigations (171.0 mg./Kg.), Table I.

The difficulty in obtaining a sharp end point and the unexplained variations in fatal dosage in this investigation and the lack of agreement of our results with those of other workers and among other investigators themselves would seem to make the guinea pig less suitable than the cat for the assay of digitalis.

SUMMARY

1. Eighty-four male guinea pigs were anesthetized with 1.0 to 1.2 grams of urethane per kilogram of body weight and subsequently injected with varying concentrations of U. S. P. Reference Standard Digitalis (1942) Tincture. The animals were arranged in seven groups of twelve each and properly randomized. They were tested with the following seven concentrations: 1.60, 2.26, 3.20, 4.53, 6.40, 9.05, and 12.80 per cent of the tincture, respectively. The dilutions were made with normal saline solution. The lethal dose of drug per kilogram of body weight was otherwise determined by the U. S. P. XIII method for assaying digitalis on cats, except that cannulation of the jugular was made instead of the femoral vein. Results from animals showing gross abnormalities of the lungs were disregarded

2. The end point was difficult to determine because the respiration became irregular considerably before the heart beats became sporadic and finally ceased.

3. No seasonal variation was demonstrable during the testing period.

4. The rate of cumulation of digitalis became more rapid with higher concentrations, hence a curved line with a negative slope seemed to fit the data best as contrasted to the straight line with the slope in the reverse direction obtained by Holck, *et al.*, in the cat for the five highest concentrations. In the present study the curve became level between 25 and 38 injections (125 and 190 minutes) indicating that lower concentrations prevent early cumulation of digitalis.

5. To ascertain whether the high alcoholic content of the stronger concentrations of the tincture were in part responsible for the lower lethal doses, a comparison between the U. S. P. Reference Standard Digitalis Tincture (4.53 per cent) and the same tincture with added alcohol was made. No significant difference in toxicity was produced by such increased alcoholic content. However, for unexplained reasons the mean lethal dose obtained for the control group in the alcohol comparison (148.8 mg./Kg.) was significantly lower than the mean lethal dose obtained in the main study with the same concentration (171.0 mg./Kg.).

6. Concentration-lethal dose correlation was poor when compared with the results of Allmark and Bachinski (3) who also used urethane anesthesia and with results of other investigators using different anesthetics.

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The Efficacy of Cetyl Trimethyl Ammonium Bromide in a Series of Ointment Bases*

By LEONARD P. PRUSAK† and ALBERT M. MATTOCKS‡

The efficacy of Cetyl Trimethyl Ammonium Bromide in a series of ointment bases has been tested and the results obtained are reported. Of the bases tested, those which were found to be most satisfactory are pectin paste and a base prepared by mixing a polyethylene glycol ("Carbowax") with water.

MUCH interest has been focused in recent years on a new group of synthetic agents collectively known as quaternary ammonium salts. They reportedly possess powerful germicidal effectiveness and are widely used at present in medicine and industry as germicides and preservatives.

Although much evidence has accumulated which demonstrates the powerful antibacterial action of quaternary ammonium salts in the form of tinctures and aqueous solutions, there is little knowledge regarding their activities in ointment form. Recently Hart and Huyck (1) evaluated Hyamine-X in five different ointment bases, using the wet and dry filter paper methods and the agar cup-plate method of assay (2). These workers concluded that Hydrophilic Ointment modified by removal of the cationic agent was the most suitable of the bases tested.

The purpose of this study was to investigate the bactericidal powers of quaternary ammonium salts in a representative series of ointment bases and to learn whether the ointment bases released the medicament in active form. For initial studies a single quaternary ammonium salt, Cetyl Trimethyl Ammonium Bromide (Baker), CTAB, was selected as a typical compound.

Previous investigations have shown that bacteriological evaluations of quaternary ammonium salts give abnormally high phenol coefficients by the FDA dilution method (3) and inconsistent results by the agar cup-plate method (4). These methods, therefore, were not considered suitable for the testing of CTAB ointments.

The quaternary ammonium salts are such powerful bacteriostatic agents that a loopful of broth used for subcultures may contain sufficient medicament to cause bacteriostasis and thus give erroneous results. To overcome this difficulty, several methods have been suggested, such as that of Klarman and Wright (5) which utilizes high dilution technique; that of Lawrence (6) which uses Suramin as an inactivating agent; and that of Quisno, Gibby, and Foter (7) which uses lecithin as an inactivator. The latter workers found that a broth containing lecithin in predetermined amounts dispersed with one-half per cent Tween-80 could be used as a subculture medium which would prevent further action of quaternary ammonium salt on surviving bacteria. This medium was named Letheen Broth.

In this study the CTAB ointments were evaluated by dispersing them in broth inoculated with *Staphylococcus aureus*, incubating the mixtures at 37° for periods of three and twenty-four hours, and then subculturing a loopful of each into Letheen Broth.

An unsuccessful attempt was made to determine the amounts of CTAB released into broth by the various ointments using the colorimetric assay of Auerbach (8, 9).

A bacteriological experiment was conducted to show the effect of blank ointment bases upon effective concentrations of CTAB.

EXPERIMENTAL

Five ointment bases official in the United States Pharmacopœia XIII and the National Formulary VIII and one unofficial base were used in this investigation. Hydrophilic Ointment U. S. P., a typical oil-in-water emulsion base, Hydrophilic Petrolatum U. S. P., a water-in-oil emulsion base, Simple Ointment U. S. P., a petrolatum-type base, Tragacanth Glycerite N. F. and Pectin Paste N. F., mucilaginous bases, and Carbowax Ointment Base (prepared by mixing Carbowax 1500 with 10% water), a polyethylene glycol base, were selected as a representative group of ointment bases.

The ointments were prepared by incorporating 1.0 Gm. CTAB into a sufficient quantity of base to make 100 Gm. ointment, using gentle heat or a small amount of water when desirable.

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Bacteriological Assay.—The method adopted for this investigation was one involving the inactivation of quaternary ammonium salts by lecithin.

In the bacteriological tests a 0.5 Gm. sample of ointment containing 1% CTAB was to be used in a total mixture of 6 Gm. It was calculated that one loopful (0.05 ml.) of this mixture would contain at most 4.1×10^{-5} Gm. of CTAB. When transferred to the subculture medium, the maximum concentration of CTAB would become 1:120,000. In order to prove that the Letheen Broth used in the subcultures was sufficient to inactivate the maximum amount of quaternary salt which might be present, the following experiment was performed: One ml. of 1:11,000 CTAB was added to each of six tubes containing 5 ml. Letheen Broth (0.07% vegetable lecithin) and 0.5 ml. of a 24-hour culture of *S. aureus* (10), resulting in a final CTAB concentration of about 1:71,000. The mixture was incubated twenty-four hours at 37° and read for turbidity. A parallel series was run using nutrient broth that did not contain lecithin. All tubes containing lecithin showed bacterial growth while all those without lecithin were growth-free. This demonstrated that the Letheen Broth effectively neutralized the maximum amounts of CTAB that might be present in subcultures.

The technique employed for antibacterial testing of ointments was as follows:

Tubes containing 5 ml. quantities of sterile nutrient broth were inoculated with 0.5 ml. of a twenty-two- to twenty-six-hour culture of *S. aureus*. One-half gram of 1% CTAB ointment, accurately weighed on high gloss paper, was added to the inoculated broth and the mixture incubated for three-hour and twenty-four-hour periods at 37°. At the end of each incubation, 4-mm. loopfuls were subcultured into 5-ml. Letheen Broth, shaken thoroughly with a hand-operated shaking machine and incubated for forty-eight hours. The tubes were then read for turbidity. Results are shown in Table I.

TABLE I.—GERMICIDAL EFFECTIVENESS OF 1% CTAB OINTMENTS[a, b]

Ointment (Containing 1% CTAB)	Bacterial Growth			
	3-Hr. Incubation		24-Hr. Incubation	
Hydrophilic Ointment	+++	+++	+++	+++
Hydrophilic Petrolatum	+++	+++	+++	+++
Simple Ointment	+++	−−−	+−−	+++
	−−+		−−−	−−−
			−−−	−−−
			−−+	
Tragacanth Glycerite	−−−	−−+	−++	−−+
	−−−	+++		
Pectin Paste	−−−	−−−	−−−	−−−
Carbowax Ointment	−−−	−−−	−−−	−−−
	−−−	−−−		

[a] + indicates turbidity (growth); − indicates no turbidity.

[b] Each symbol represents one test.

Interference Tests.—To obtain a more accurate picture of the germicidal activity of CTAB in ointment bases, it became necessary to determine the extent of interference, if any, imparted by the various ointment bases. To accomplish this, bacteriological tests were carried out in the following manner:

Analytically accurate dilutions of CTAB ranging in concentration from 1:5000 to 1:140,000 were prepared and tested for bactericidal activity. A 1-ml. quantity of each dilution was added to a tube containing 5 ml. nutrient broth and 0.5 ml. of a twenty-four-hour culture of *S. aureus*, and the mixture was incubated for twenty-four hours. At the end of this time 0.05 ml. of the mixture was subcultured in 5 ml. Letheen Broth, and this was incubated for forty-eight hours. A second series of tubes containing the same amounts of CTAB was run under the same conditions except that 0.5 Gm. of the blank ointment base was added after the CTAB had been thoroughly mixed with the broth and before the mixture was inoculated with bacteria. Corresponding results were obtained by a duplication of the experiment. Results are shown in Table II.

Attempted Assay of Released Germicide by Colorimetric Procedure.—Ointments were added to broths in the usual way, and, after suitable incubation, were assayed by the colorimetric method of Auerbach. Results were inconsistent with bacteriological findings, and further investigation demonstrated that the ointment bases adsorbed in varying degrees the CTAB-bromphenol colored complex. This explained the erratic colorimetric results and proved that the Auerbach method is not suitable for ointments without special modifications to overcome adsorption effects.

DISCUSSION

The experimental work was planned to show: (1) the relative efficacies of six ointments containing 1% CTAB, (2) whether decreased effectiveness of the germicide contained in the ointments was due to failure of the base to release the medicament. In order for an ointment to be considered efficient by this method of evaluation, it must be capable of releasing the germicidal agent into nutrient broth. Also, the ointment base itself must not interfere with or inhibit the action of already-released CTAB.

The bactericidal tests (Table I) showed clearly the relative efficacies of the six ointments tested by demonstrating the bactericidal action after three and twenty-four hours' contact with the bacteria. These results represent a summation of the various physical and chemical properties of the ointment bases that affect the activity of CTAB on bacteria.

The interference measurements (Table II) showed the maximum dilution of CTAB free of ointment base that had bactericidal activity as compared with the same dilutions when mixed with ointment bases. This, in effect, was a direct measurement of the ability of the base to remove CTAB from solution, by adsorption or precipitation, or to prevent, in some way, the action of CTAB on bacteria.

Hydrophilic Ointment U. S. P. showed no bactericidal activity in spite of prolonged contact with bacteria. The interference measurements showed that this ointment base prevented the antibacterial action of even the most concentrated solution of CTAB tested. Since this ointment is an oil-in-water emulsion containing the germicidal compound in the aqueous phase, it may be assumed that the CTAB molecules should have ample opportunity to come in

TABLE II.—INTERFERENCE TESTS[a, b]

Conc. CTAB Added	Without Ointment Base	With Ointment Bases Added					
		Hydrophilic Ointment	Hydrophilic Petrolatum	Simple Ointment	Tragacanth Glycerite	Pectin Paste	Carbowax Ointment
1:5000	—	M	M	S	—	—	—
1:10,000	—	M	M	S	S	—	—
1:20,000	—	H	H	S	S	—	S
1:40,000	S	H	H	M	M	S	S
1:60,000	S	H	H	M	H	S	M
1:80,000	M	H	H	M	H	M	M
1:100,000	M	H	H	H	H	M	H
1:120,000	H	H	H	H	H	H	H
1:140,000	H	H	H	H	H	H	H

[a] Each symbol represents a duplicate set of tests.
[b] S = Slight growth; M = Moderate growth; H = Heavy growth; — = No growth.

contact with the bacteria, other factors not being considered. However, it is known that sodium lauryl sulfate (which is present in Hydrophilic Ointment) forms a precipitate with CTAB in aqueous solution, and it is possible that this phenomenon is responsible for the lack of activity of CTAB in this base. This view is substantiated by the results of Hart and Huyck (1) which showed that Hydrophilic Ointment without sodium lauryl sulfate released effective amounts of a quaternary ammonium germicide.

Hydrophilic Petrolatum U. S. P. also gave negative bactericidal results, and prevented CTAB activity in the interference tests. There is no indication that a specific chemical reaction takes place between CTAB and any of the ingredients of the ointment, such as occurs with sodium lauryl sulfate in Hydrophilic Ointment. However, this base has a powerful inactivating or adsorption effect, as shown by interference tests, and may not be considered suitable as a vehicle for CTAB.

Simple Ointment U. S. P. showed bactericidal activity in about 50% of the three-hour tests and in about 80% of the twenty-four hour tests. This makes it appear that the amount of CTAB released from the ointment gives a concentration of germicide very near the minimum effective range. Interference measurements substantiate the bactericidal tests, since the ointment base inactivated CTAB to such an extent that slight bacterial growth was noted with the three highest concentrations and moderate to heavy growth with lower concentrations of germicide. These results indicate that Simple Ointment has an adsorptive or inactivating power for CTAB which prevents release of the latter in effective amounts.

Tragacanth Glycerite N. F. proved to be of doubtful effectiveness by bactericidal evaluations. Interference measurements indicated that the base prevents the action of CTAB in concentration of 1:60,000 or less. If all of the germicide contained in the ointment had been released into the broth, the resultant concentration would have been 1:1200. It is apparent, therefore, that most of the CTAB remained adsorbed on the ointment base, leaving in solution a noneffective concentration.

Pectin Paste N. F. proved to be an effective carrier of CTAB. It had bactericidal action on both three-hour and twenty-four hour tests. Interference measurements proved that this ointment interfered in no way with action of the germicide, a solution containing ointment being equally as effective as an aqueous solution of germicide alone. This makes it appear that Pectin Paste is an ideal base for CTAB ointment.

Carbowax Ointment had bactericidal properties in both three and twenty-four hour tests. Interference measurements indicated that Carbowax does prevent the action of or adsorb CTAB to a very limited extent. For all practical purposes, it is believed that Carbowax is as valuable a carrier of CTAB as Pectin Paste.

CONCLUSIONS

Hydrophilic Ointment U. S. P. and Hydrophilic Petrolatum U. S. P. are not satisfactory carriers of CTAB. Simple Ointment U. S. P. and Tragacanth Glycerite N. F. release such small amounts of quaternary ammonium salt from their combinations that they cannot be considered useful bases for CTAB.

Pectin Paste N. F. and Carbowax Ointment readily release CTAB in an active form, demonstrating that they are suitable bases for quaternary ammonium salts.

The method of bactericidal evaluation of quaternary ammonium compounds devised by Quisno, Gibby, and Foter can be conveniently and satisfactorily applied to ointments.

The Auerbach Method of analysis of quaternary ammonium salts is not satisfactory for the assay of ointments.

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The Synthesis of Pyridine Derivatives Possessing Possible Emetic and Rodenticidal Activity*

By WILLIAM D. JONES,† GLENN L. JENKINS, and JOHN E. CHRISTIAN

Since the methoxy group is present in emetine, in a patented emetic, and in several nauseant benzene derivatives it is possible that this group may be responsible for the emetic action of these compounds. In this report the syntheses of several methoxy derivatives of pyridine are described.

A RODENTICIDE which also possessed emetic properties would be considerably more advantageous than the usual rat poison which is equally fatal to higher animals. Its advantage would be in the fact that rats cannot regurgitate while other animals can. Hence, if the emetic-poison were taken by household pets, such as dogs or cats, the poison would promptly be regurgitated, but to rats, which do not have this power, it would be fatal.

Since the methoxy group is present in emetine (an alkaloid which can cause emesis by irritating the stomach mucosa), in a patented emetic consisting of 6,7-dimethoxyisoquinoline (1) and in several nauseant methoxy benzene derivatives prepared by some German investigators seeking antimalarials (2), it would appear that the methoxy group is responsible for the emetic activity exhibited by these compounds.

On the basis of this information, it was decided to investigate the influence of the methoxy group on the pyridine nucleus. It was hoped that the addition of the methoxy group to pyridine would lend emetic properties and with such toxic groups as chlorine, produce a product which might be a useful rodenticide.

EXPERIMENTAL

1. 5-Chloro-2-aminopyridine and 3,5-dichloro-2-aminopyridine.—The procedure used was that of Chichibabin and Egorov (3).

2. The attempted synthesis of 5-methoxy-2-aminopyridine.—A series of experiments was performed in an attempt to prepare 5-methoxy-2-aminopyridine from 5-chloro-2-aminopyridine by modification of the procedure of Schickh, Binz, and Schulz (4). The following is representative of these experiments which yielded about 45–50% of a colorless oil. The other experiments (using lower temperatures and pressures and altering the reactions) yielded a small quantity of this oil and large amounts of starting materials. One hundred grams of 5-chloro-2-aminopyridine, 130 Gm. solid sodium methoxide (95%), 500 ml. methanol, and 25 Gm. copper sulfate were charged to the steel liner of a pressure bomb. The liner was placed in the bomb and heated under an atmosphere of nitrogen at 300° for three hours. After cooling, the contents were removed from the liner, filtered, the solvent removed under vacuum, the residue treated with a little water and extracted with ether. The ether was removed under vacuum and the residue distilled using vacuum. The product was a colorless oil boiling at 203–204° (at atmospheric pressure).

Anal.: Calcd. for $C_6H_8N_2O$: N, 22.6%; C, 58.0%; H, 6.45%. Found: N, 25.0%; C, 66.0%; H, 7.48%.

The analysis clearly indicated that the compound sought was not obtained. When the analysis, boiling point, and the melting point of the picrate of this compound and 2-methylaminopyridine were compared, it was concluded that the compound prepared by this procedure was 2-methylaminopyridine.

	Unknown	2-Methylaminopyridine
Carbon	66.0%	66.7%
Hydrogen	7.48%	7.42%
Nitrogen	25.0%	25.9%
Boiling point	203–204°	200–201° (10)
Melting point of picrate	192°	190° (10)

3. 5-Chloro-3-nitro-2-aminopyridine.—The procedure followed was that of Chichibabin and Egorov (3).

4. 5-Chloro-3-nitro-2-bromopyridine.—Craig's procedure (5) for the preparation of 2-bromopyridine from 2-aminopyridine was followed. Five grams of 5-chloro-3-nitro-2-aminopyridine was dissolved in 14 ml. of cold hydrogen bromide (48%). Four and three-tenths grams of bromine was cautiously added to the stirred reaction mixture. A solution of 4.9 Gm. of sodium nitrite in 7 ml. of water was added dropwise and the mixture stirred for two hours. Ten and nine-tenths grams of sodium hydroxide in 25 ml. of water was added causing a precipitate to form. The precipitate was filtered off, dissolved in alcohol, decolorizing charcoal added, the charcoal removed by filtration, and the filtrate chilled. The product, yellow tinged white plates, melted at 77–79°. The yield was 14.6% of theoretical.

Anal.: Calcd. for $C_5H_2BrClN_2O_2$: N, 11.8%. Found: N, 11.8%.

5. 5-Chloro-3-nitro-2-hydroxypyridine.—The procedure used was a modification of that used by Phillips (6) for preparing 5-nitro-2-hydroxypyridine. Twelve grams of 5-chloro-3-nitro-2-aminopyridine was dissolved in 100 ml. water and 50 ml.

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sulfuric acid. This solution was chilled and 10 Gm. sodium nitrite in 20 ml. of water was added dropwise with vigorous stirring. The product precipitated out during the addition of the sodium nitrite solution. The crystals were filtered off, washed with water, and dried. The product consisting of light yellow needles weighed 10.3 Gm. (85.5% of theoretical yield) and melted at 231–234°. The melting point was not changed after several recrystallizations from benzene and alcohol.

Anal.: Calcd. for $C_6H_4ClN_3O_3$: N, 16.1%. Found: N, 15.8%.

Several attempts to reduce catalytically 5-chloro-3-nitro-2-hydroxypyridine in different solvent combinations using a Parr Catalytic Hydrogenation apparatus produced only tarry residues.

6. 5 - Chloro - 3 - nitro - 2 - methoxypyridine.—The procedure followed was a modification of the method developed by Bickel (7) for preparing methoxypyridine derivatives. Twenty-five grams of 5-chloro-3-nitro-2-hydroxypyridine was suspended in 100 ml. of benzene. To the cooled and stirred suspension, 17.5 Gm. of diazomethane in 637 ml. of benzene was added slowly. The reaction mixture was allowed to stand overnight at room temperature. The solvent was removed under vacuum and the residue was purified by sublimation (15 mm. pressure). The product was 16.8 Gm. (62.3% of theoretical) of pale yellow needles melting at 84–88°.

Anal.: Calcd. for $C_6H_5ClN_2O_3$: N, 14.9%. Found: N, 14.6%.

When 5-chloro-3-nitro-2-methoxypyridine was reduced catalytically (in a Parr Catalytic Hydrogenation apparatus), white needles melting at 168–170° were obtained after purification by sublimation.

Anal.: Calcd. for $C_6H_7ClN_2O$: N, 17.7%; C, 45-6%; H, 4.4%. Found: N, 21.8%; C, 49.5%; H, 5.0%

The analysis indicated that the product formed was not 5-chloro-3-amino-2-methoxypyridine. The presence of a primary amine could not be shown. The product remained unidentified.

7. The attempted synthesis of 3,5-dimethoxy-2-aminopyridine.—Three attempts were made to react 3,5-dichloro-2-aminopyridine with sodium methoxide at high temperatures and pressures in a pressure bomb using copper sulfate as a catalyst, but no product was attainable.

8. The synthesis of 3,5-dibromopyridine.—The procedure followed was that of McElvain and Goese (8). Four attempts (modifications of the procedure of Koenigs, *et al.* (9), were made to react 3,5-dibromopyridine with sodium methoxide using copper sulfate as the catalyst in a pressure bomb. The reaction conditions tried were as follows: Ten hours at 200°, twenty hours at 200°, three hours at 250°, and one hour at 300°.—No product was attainable.

9. The attempted synthesis of 5-diethylamino-2-aminopyridine.—Ten grams of 5-chloro-2-aminopyridine, 17 Gm. of diethylamine, 10 Gm. copper sulfate, 5 Gm. sodium carbonate, and 500 ml. of benzene was charged to the steel liner of the pressure bomb. The liner was placed in the bomb, nitrogen added, and the bomb heated at 250° for seven hours. After cooling, the contents of the liner were removed, filtered, the solvent removed under vacuum, and the residual oil treated with water. The mixture was extracted with ether, the ether removed under vacuum, and the residue vacuum distilled. The product which distilled at 100–103° (16 mm. pressure) crystallized upon cooling and was recrystallized from ligroin. The material was white plates melting at 60°.

Anal.: Calcd. for $C_9H_{13}N_3$: N, 25.5%; C, 65.5%; H, 9.0%. Found: N, 29.7%; C, 64.5%; H, 6.9%.

The analysis proved that the product was not 5-diethylamino-2-amino-pyridine but indicated that the compound 2,2′-diamino 5,5′-dipyridyl was formed by the reaction.

Anal.: Calcd. for $C_{10}H_{10}N_4$: N, 30.1%; C, 64.1%; H, 5.4%. Found: N, 29.7%; C, 64.4%; H, 6.9%.

The proof that 2,2′-diamino 5,5′-dipyridyl was the product formed was shown by the preparation of the dibenzenesulfonamide, the tetranitro derivative, the dipicrate and through the synthesis of the compound by heating 5-chloro-2-aminopyridine with sodium carbonate and copper sulfate in the pressure bomb.

10. 2,2′-Dibenzenesulfonamide 5,5′-dipyridyl.—Eight-hundredths of a gram of 2,2′-diamino 5,5′-dipyridyl was heated on a steam bath with 1 ml. of benzenesulfonyl chloride. Five milliliters of 10% sodium hydroxide was added and the heating continued for several more minutes. The sodium hydroxide layer was decanted and neutralized. The crystalline product which precipitated was recrystallized from benzene. The product melted at 173–174°.

Anal.: Calcd. for $C_{22}H_{18}N_4O_4S_2$: N, 12.0%. Found: N, 12.2%.

11. Tetranitro 2,2′-diamino 5,5′-dipyridyl.—Five-tenths of a gram of the 2,2′-diamino 5,5′-dipyridyl was dissolved in 6.6 ml. of cold concentrated sulfuric acid. Five-tenths of a milliliter of concentrated nitric acid was added dropwise with stirring to the solution. After standing one-half hour at room temperature, the solution was poured into ice water. The precipitate which formed was filtered off, washed and recrystallized from 95% ethanol. The product melted at 164° (dec.) and consisted of yellow plates.

Anal.: Calcd. for $C_{10}H_6N_8O_8$: C, 32.8%; N, 30.6%; H, 1.6%. Found: C, 32.9%; N, 30.7%; H, 2.4%.

12. The dipicrate of 2,2′-diamino 5,5′-dipyridyl.—About 0.05 Gm. of 2,2′-diamono 5,5′-dipyridyl was dissolved in 5 ml. of 95% ethanol and heated on a steam bath. Saturated picric acid in ethanol was added, the solution cooled, and the crystals filtered off. The product, recrystallized from ethanol, melted at 244–246° (dec.).

Anal.: Calcd. for $C_{22}H_{14}N_{10}O_{14}$: N, 21.7%. Found: N, 21.7%.

13. 2,2′-Diamino 5,5′-dipyridyl.—In gathering additional proof that the product in Experiment 9 was 2,2′-diamino 5,5′-dipyridyl, 15 Gm. of 5-chloro-2-aminopyridine was heated with 18.0 Gm. sodium carbonate, 7.0 Gm. copper sulfate, and 150 ml. benzene at 300° for three hours in a pressure bomb. The product was shown to be identical with that in Experiment 9 by a mixed melting point. 2,2′-Diamino 5,5′-dipyridyl can also be prepared by heating 5-chloro-2-aminopyridine with sodium ethoxide and copper sulfate at 300° for three hours

The yields were about 1.5% of theoretical in both experiments.

14. 3,5-Dichloro-2-hydroxypyridine.—The procedure used was that of Chichibabin and Egorov (3).

15. 3,5 - Dichloro - 2 - methoxypyridine.—Seven grams of 3,5-dichloro-2-hydroxypyridine was suspended in 50 ml. of ether. One hundred and five milliliters of an ether solution containing about 5.4 Gm. of diazomethane was added slowly to the suspension. The reaction mixture was allowed to stand overnight. The solvent was removed under vacuum and the residue recrystallized from carbon tetrachloride. The product, white needles, weighed 5 Gm. (66.2% of theoretical) and melted at 137–140°.

Anal.: Calcd. for $C_6H_5Cl_2NO$: N, 7.9%. Found: N, 7.86%.

16. 5-Chloro-2-hydroxypyridine.—The procedure followed was that of Chichibabin and Egorov (3).

17. 5-Chloro-2-methoxypyridine.—Six grams of 5-chloro-2-hydroxypyridine was suspended in 50 ml. of benzene. One hundred and fifty-five milliliters of an ether solution containing about 5.7 Gm. of diazomethane was added slowly with stirring to the suspension. The reaction mixture was allowed to stand overnight. The solvent was removed and the residue (an oily crystalline mass) was crystallized from ligroin and benzene. The product, white needles, weighed 3 Gm. (45.2% of theoretical) and melted at 45–46°.

Anal.: Calcd. for C_6H_6ClNO: N, 9.85%. Found: N, 9.70%.

SUMMARY

1. Three chloro-methoxypyridine derivatives possessing possible emetic and rodenticidal activity have been synthesized, namely: (*a*) 5-chloro - 3 - nitro - 2 - methoxypyridine; (*b*) 3,5-dichloro - 2 - methoxypyridine; (*c*) 5 - chloro-2-methoxypyridine.

2. The following other new compounds have been synthesized: (*a*) 5-chloro-3-nitro-2-bromopyridine; (*b*) 5 - chloro - 3 - nitro - 2 - hydroxypyridine; (*c*) 2,2′ - diamino 5,5′ - dipyridyl; (*d*) 2,2′ - dibenzenesulfonamide 5,5′ - dipyridyl; (*e*) tetranitro 2,2′-diamino 5,5′-dipyridyl; (*f*) 2,2′ - diamino 5,5′ - dipyridyl dipicrate.

3. When the three methoxy compounds were given a preliminary test for possible action on the emetic center in the medulla (using pigeons), 5-chloro-2-methoxypyridine caused emesis in one-half hour, 3,5-dichloro-2-methoxypyridine was toxic but caused no emesis and the solubility of 5-chloro-3-nitro-2-methoxypyridine prevented a complete test (small doses caused no emesis).

4. Quantities of both 5-chloro-2-methoxypyridine and 3,5-dichloro-2-methoxypyridine are available for further pharmacological testing.

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Solubility of Cinchonine and Cinchonidine in Alcohol-Water Mixtures*

By NAZIR A. CHOWDHRY and CHARLES F. BONILLA

The solubility of cinchonine and of cinchonidine in mixtures of alcohol and water in different proportions has been determined. The method employed and the results obtained are reported.

IN CONNECTION with further development work at this University of the U. S. Army portable quinine extraction plant (1), sponsored by the Department of Commerce and Reconstruction Finance Corporation, it was desirable to know the solubility of the cinchona alkaloids in alcohol-water mixtures at room and higher temperatures. These data would be useful in predicting the amount of alcohol necessary to extract the alkaloids from the Zeo-Karb bed, the amount remaining in solution in the alcohol recovery still product liquor, and the effect of varying the alcohol concentration and temperature on both of these quantities.

EXPERIMENTAL

The Ecuadorean bark employed in testing the process analyzed 1.0% quinine, 1.3% cinchonidine,

* Received April 21, 1948, from the Department of Chemical Engineering, Johns Hopkins University, Baltimore, Md.

and 2.0% cinchonine. In view of the smaller amount and much higher solubility in alcohol reported for quinine (2) only the solubilities of cinchonine and cinchonidine were believed to be necessary.

A literature survey revealed no data on intermediate alcohol concentrations, and discrepancies and uncertainties among the available figures for concentrated alcohol, as indicated in Table I.

low, though saturation seemed to have been attained as completely as at the other conditions.

To obtain approximate solubilities at other temperatures, the experimental values were recomputed to percentage of the weight of solvent, and the logarithm of this quantity plotted against the reciprocal of the absolute temperature. Due to the low concentration (in mole fraction) of these solutions, this plot should yield a straight line (2). A straight

TABLE I.—PREVIOUS SOLUBILITY DATA IN ALCOHOL

Alkaloid	Temp., ° C.	Alcohol Concentration	Solubility, % of Total Weight	Reference
Cinchonine	25° ±	96.6% ± by vol. (or 100%?)	2.04	(3)
Cinchonine	15° ±	96.6% ± by vol. (or 100%?)	0.826 (or 1.02?)	(4)
Cinchonine	20°	88.8% by vol. (S.G. 25° = 0.832)	0.855	(5)
Cinchonidine	25° ±	96.6% by vol. (or 100%?)	4.76	(3)
Cinchonidine	15° ±	96.6% by vol. (or 100%?)	4.76 (or 5.8?)	(4)

Uncertainties in alcohol concentration indicated in Table I are due to its not being stated definitely in the references cited, and in solubility to no statement as to whether volume or mass of the solvent is intended. Accordingly the desired solubilities of commercial samples of pure cinchonine and cinchonidine were determined.

An excess of the dried alkaloid was shaken frequently in tightly capped bottles with the desired composition of alcohol until 3- or 5-ml. portions of the liquid, pipetted out at about fifteen-minute intervals, weighed, and evaporated to dryness in a weighing bottle in air at 50°, showed a constant percentage of residue. Each experimental figure reported in Table II was obtained in at least two consecutive check determinations, indicating saturation

line was therefore drawn through each pair of points for the same solute and solvent, and the solubility at each desired temperature read off. These figures were recomputed to per cent based on the total weight and are listed also in Table II. The accuracy is probably least at the highest and lowest temperatures given, but should be fairly good since the lines for both alkaloids were nearly parallel, and what variation in slope occurred took place gradually in passing from one alcohol concentration to the next (except for cinchonine in 50% alcohol, which therefore was not extrapolated).

Comparing Table I with Table II it is seen that previous solubility values for cinchonine are higher than the new ones, whereas for cinchonidine they are lower.

TABLE II.—SOLUBILITY OF ALKALOIDS IN ALCOHOL-WATER MIXTURES

% by Volume of Alcohol in Solvent	Solubility of Cinchonine in Gm. per 100 Gm. of Solution, at ° C.										
	0	10	15	20	25	28.2	30	40	50	60	70
96.6	0.517	0.681	0.775	0.803	0.99	1.08	1.13	1.41	1.73	2.12	2.58
90	0.394	0.54	0.626	0.725	0.833	0.894	0.946	1.22	1.54	1.91	2.37
80	0.19	0.28	0.339	0.418	0.493	0.542	0.577	0.804	1.09	1.45	1.91
70	0.0885	0.135	0.165	0.20	0.239	0.271	0.286	0.404	0.56	0.754	1.02
60	0.049	0.075	0.092	0.114	0.136	0.153	0.163	0.23	0.32	0.433	0.586
50	...	...	...	...	...	0.0304	...	...	0.15	....	....
	Solubility of Cinchonidine in Gm. per 100 Gm. of Solution at ° C.										
	0	10	15	20	25	27.9	30	40	50	60	70
96.6	3.85	4.97	5.59	6.29	7.06	7.52	7.83	9.42	11.51	13.6	16.0
90	3.48	4.55	5.17	5.84	6.54	7.02	7.34	9.08	11.02	13.0	15.5
80	1.96	2.7	3.15	3.61	4.17	4.54	4.75	6.1	7.71	9.54	11.8
70	0.868	1.26	1.52	1.81	2.15	2.35	2.5	3.4	4.55	5.84	7.58
60	0.46	0.70	0.853	1.04	1.26	1.38	1.49	2.09	2.89	3.85	5.12
50	0.097	0.159	0.202	0.255	0.316	0.36	0.391	0.585	0.88	1.22	1.73

had been reached. For the 50° tests a Carrier atmospheric drying cabinet, automatically maintained within less than 0.5° of this temperature, was employed.

The results are listed in Table II under 27.9°, 28.2°, and 50° C. If these solubilities are plotted as isotherms against the solvent composition, quite smooth curves result. However, the value for cinchonine at 28.2° in 50% alcohol appears to be

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Chemical Constitution and Analgetic Action*

By F. W. SCHUELER, E. G. GROSS, and H. HOLLAND

Recent advances in the pharmacology of morphine and its analogs suggest that at least part of the analgetic response of these agents is traceable to their action through the autonomic nervous system. Evidence derived from a consideration of the chemical constitutional relations involved in morphine and a host of new analogs support this view and indicate the presence of both sympathomimetic and parasympathomimetic moieties connected (in general) by the same amino nitrogen atom within the same molecule.

IN RECENT years it has been indicated that the definition of chemical constitutional requirements for a given type of pharmacodynamic action may be best elucidated when specific chemical constitutional aspects of a given molecule are made the focus of interpretation rather than vague references to the molecule as a whole. The successful application of this approach to the problem of sex-hormonal action (21, 22) and muscarinic action (19) has encouraged us to extend it to the elucidation of the relation between analgetic action and chemical constitution.

In spite of the common assertion that morphine owes its analgetic effect to a "sledge-hammer" central depressant action, certain facts stand in the way of a ready acceptance that this is the only mechanism involved. Some of these facts are outlined in the summary and discussion below.

1. The peak analgetic response to morphine is not simultaneous with its peak central depressant effect (18, 24).

2. Some animals show great central excitation with morphine in spite of a concomitant analgetic response. For example, the cat shows great central excitement with morphine, but requires a smaller (per kilo) dose of morphine for a given analgesic response than the dog, which is depressed by this drug (12).

3. The analgetic response to morphine is not decreased by amphetamine and other C. N. S. stimulants, but, on the contrary, is enhanced by these agents (10).

4. *In vitro* experiments indicate the doses of morphine and its analogs for the depression of brain respiration to be too large to explain their analgetic action in physiological doses upon a central depressant basis (2).

Finally, it may be said that much new evidence acquired largely through the last decade suggests that a considerable portion of the analgesic action of morphine and its analogs may be traceable to effects involving the autonomic nervous system.

Experiments by Gross, *et al.* (1,4), indicate the analgetic action of the morphine type agents may be traceable to their peripheral perineural vasoconstrictor effect though these agents show little, if any, measurable pressor activity. Other facts indicating a peripheral mechanism of analgetic action by the opiates are: The potentiation of opiate analgesia by neostigmine (26), ephedrine, and epinephrine (10); and the observation that the cholinergic depressants, scopolamine and atropine (1,4), tend to decrease both the intensity and duration of analgesia by the opiates. Pero and Buscaino (16, 17) have advanced evidence supporting an adrenergic theory of analgesia together with a cholinergic theory of pain.

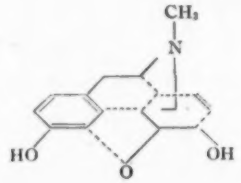
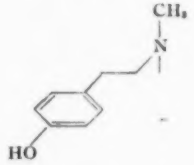
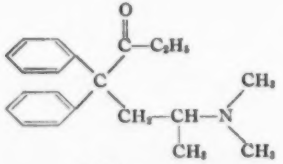
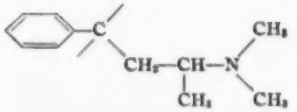
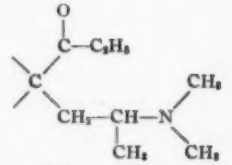
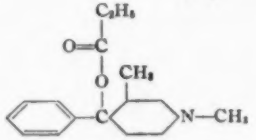
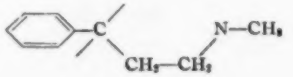
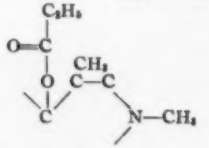
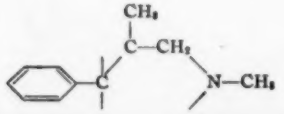
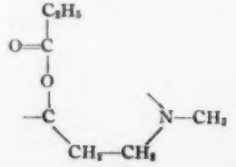
The main problem from the chemical constitutional point of view seems to involve the assumption that morphine acts both as a parasympathomimetic and a sympathomimetic agent, the extent of each such action varying in body distribution in different species, depending upon the relative sensitivity of the various physiological systems in a given species to the sympathomimetic and parasympathomimetic actions of the analgetic molecule. Evidence derived from a consideration of the chemical constitutional relations involved in morphine and its analogs indicates the presence of both sympathomimetic and parasympathomimetic moieties connected (in general) by the same amino nitrogen atom within the same molecule. The structures in Table I illustrate this generalization.

Of particular interest in connection with the notion of a parasympathomimetic moiety (muscarinic) in the analgetic molecules are some recent investigations by Pfeiffer (19, 20). Thus, for maximum muscarinic action the average optimum limiting distances between the methyl groups of the amino nitrogen to the ether-oxygen or carboxyl-oxygen, respectively, are 5.3 Å. and 7.0 Å.

Examination of the structures in Table I indicates these essential muscarinic distances to be present in the active analgetic morphine

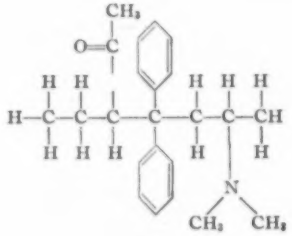
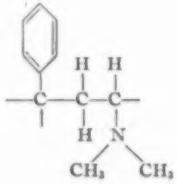
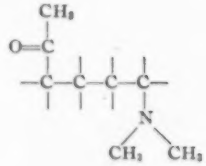
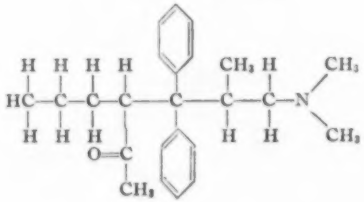
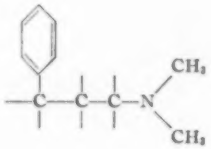
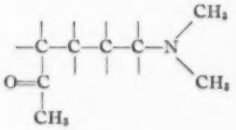
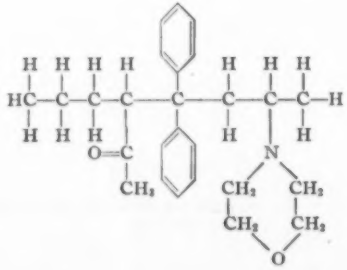
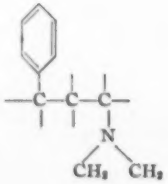
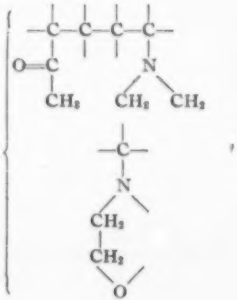
* Received June 2, 1948, from the Department of Pharmacology, College of Medicine, Univ. of Iowa, Iowa City.

TABLE I.

No. and Reference	Drug Structure	Sympathomimetic Moiety	Parasympathomimetic Moiety
1			
2 (21)			
3			
4 (8)			

(*Cont'd on p. 76*)

TABLE I.—(*Cont'd from p. 75*)

No. and Reference	Drug Structure	Sympathomimetic Moiety	Parasympathomimetic Moiety
5 (23)			
6 (23)			
7 (23)			

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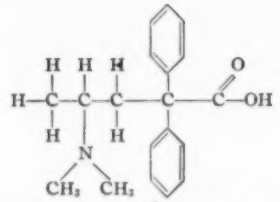

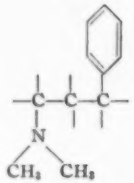

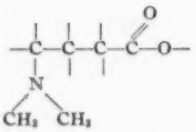

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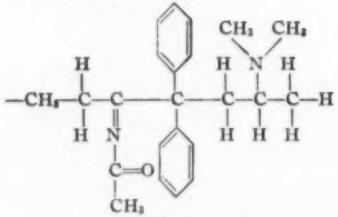
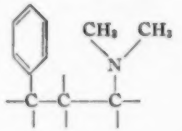
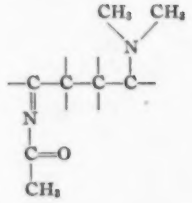
(23)







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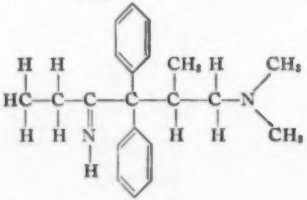

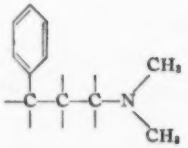

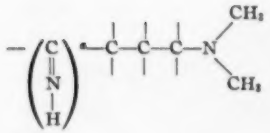

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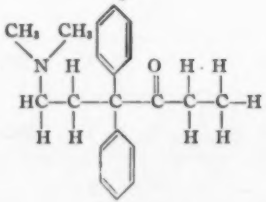
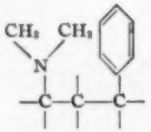
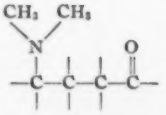


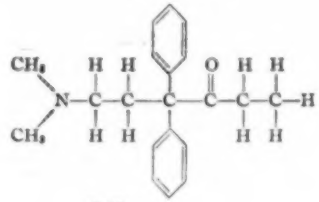
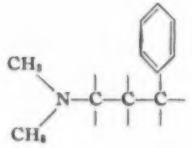
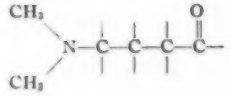
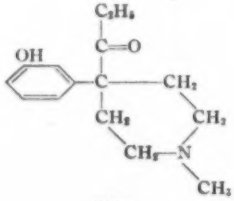
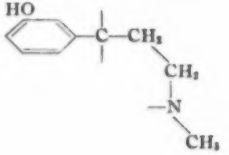
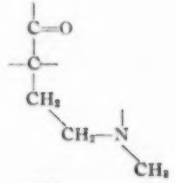
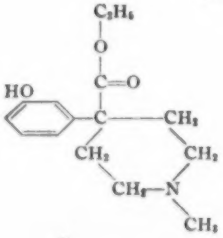
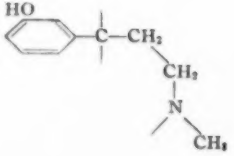
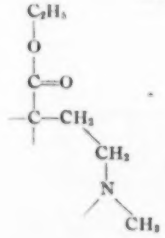
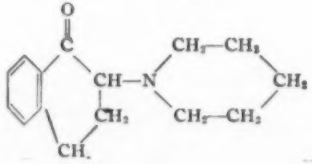
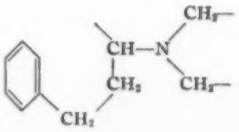
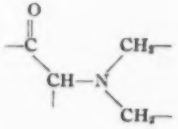




[1] From the organic chemical point of view $\rangle C = N-H$ is analogous to $\rangle C = O$ just as $-NH_2$ is to $-OH$.

(*Cont'd on p. 78*)

TABLE I.—(*Cont'd from p. 77*)

No. and Reference	Drug Structure	Sympathomimetic Moiety	Parasympathomimetic Moiety
12 (11)			
13 (11)			
14 (9)			
15 (13)			

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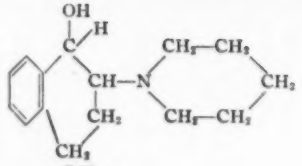

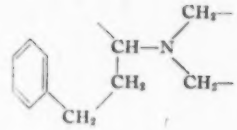

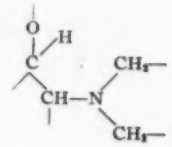

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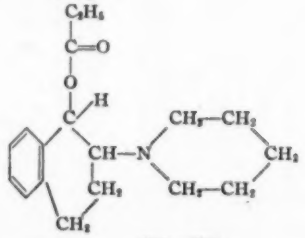

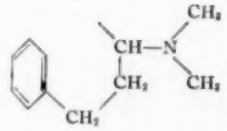

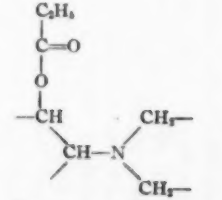

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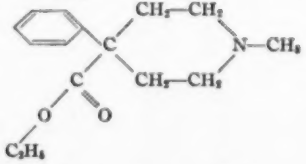

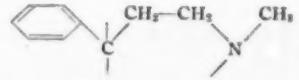

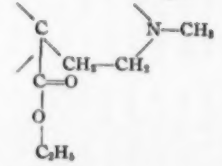

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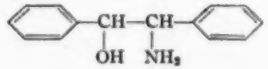
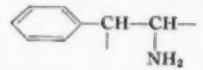
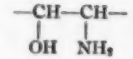
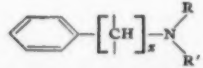


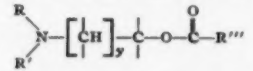




Moieties of the phenyl-alkyl-ω-amine type are termed sympathomimetic:



In general, the optimum activity of these amines with regard to sympathomimetic effect is obtained when $x = 2$ to 4.
Moieties of the tertiary amino alkyl type with terminal oxygen groups of the ether, ketone, or alcohol type are termed parasympathomimetic.



In general, the optimum activity of these amines with regard to parasympathetic effect is obtained when $y = 1$ to 3 and when *both* ketonic and ether oxygen (as in an ester) are present. When ketonic, ether, or alcoholic oxygen alone are present the parasympathomimetic effect is decreased but qualitatively similar to that of the more active analogs (17).

analogs and suggests an explanation of their parasympathomimetic type action. (See Table II.) In a similar manner, essential distances appear to function in the sympathomimetic moieties of the analgetic drug structures. Further support of these ideas is gained from analgetic tests using the Wolff-Hardy-Goodell (27) technique with white rats. Such tests show combinations of sympathomimetic agents and parasympathomimetic agents to act additively and possibly synergistically in certain dosage ranges. These effects are illustrated in Table III.

Finally, it is suggested that the analgetic action of the morphine type agents may be traceable to the presence of both sympathomimetic and parasympathomimetic moieties in the same molecule which act physiologically through a peripheral analgetic mechanism. The physiological mechanism may be a peripheral perineural vasoconstrictor effect as outlined by Gross (1,4). Recent evidence (6) has been reported to the effect that adrenectomized rats show less analgesia from morphine than normal rats and that this loss in morphine analgesia is due to the loss of epinephrine output by the adrenal medulla. These investigations emphasize the possible importance of peripheryl mechanisms in analgesia and support the notions advanced by Buscaino and Pero as to the pre-eminence of autonomic components in the analgetic process.

The parasympathomimetic portion of the analgetic molecule may be active in stimulating the output of epinephrine by the adrenal gland (3, 7), either by its direct action or through the inhibitory action upon cholinesterase, and the sympathomimetic portion of the molecule may enhance the peripheral perineural vasoconstrictor response of the epinephrine by either its direct action or by preserving epinephrine. Thus, a sympathomimetic moiety alone will exhibit some analgetic effect but when this is coupled with the epinephrine output, through stimulation of the adrenal gland by the parasympathomimetic moiety, the total analgesic effect will be maximal. Another important consideration is the fact that the powerful analgesics show a sedative effect reaching a peak which is not simultaneous with the peak analgetic response, and this sedative effect must be an expression of the constitutional relationships involved in this group of substances. The fact that analgesia reaches its peak at a different time than sedation may be explainable on the basis that the sites of analgetic drug action are different from the sites of sedative action. Indeed, the diphasic-stimulant and depressant properties (as in the cat or dog) of the powerful morphine analogs may be correlated with the relative sensitivity of the central

TABLE II.

Compound No.	x	Sympathomimetic Distance[a]	y	Parasympathomimetic Distance[b]	
1	2	5.0	2	6.0	
2	3	5.5	3	7.0	
3	3	5.5	2	7.0	C=O
				5.0	−O−
4	3	5.5	2	7.0	C=
				5.0	−O−
5	3	6.3	3	8.0	
6	3	6.3	3	8.0	
7	3	6.3	3-2	5.0	−O−
				8.0	=O
8	3	6.5	2	7.5	
9	3	6.5	3	9.0	
10	3	6.3	3	8.5	
11	3	6.3	2	8.0	
12	3	6.0	3	8.0	
13	3	5.5	3	8.0	
14	3	5.5	3	8.0	
15	3	6.0	1	5.5	
16	3	6.0	1	5.5	
17	3	6.0	1	5.5	−O−
				7.5	=O
18	3	5.5	2	7.5	
19	2	4.5	1	5.0	

[a] Sympathomimetic Distance denotes the average maximum distance between the first carbon on any alkyl group of the amino nitrogen atom and the aromatic carbon joining the ring to the alkyl carbon chain.

[b] Parasympathomimetic Distance denotes the average maximum distance between the first carbon on any alkyl group of the amino nitrogen atom and the terminal oxygen atom as computed by measurements using Fisher-Hirschfelder-Taylor Models (1 cm. = 1 Å.).

TABLE III.—MEAN ± STANDARD ERROR OF THE RISE IN ANALGETIC RESPONSE

Drug[a]	Dosage in Mg./Kg.	10 Min. After Injection	20 Min. After Injection
Ephedrine	4	2.7 ± 0.87	2.1 ± 0.42
Doryl	0.5	6.7 ± 1.56	3.4 ± 1.17
Ephedrine	2	−0.08 ± 0.42	−1.08 ± 0.42
Doryl	0.25	1.21 ± 0.65	2.34 ± 0.44
Actual effect of:			
Doryl	0.25		
Ephedrine	2	6.5 ± 1.25	3.5 ± 0.89
Theoretical effect of:			
Doryl	0.25	1.13 ± 0.76	2.26 ± 0.6
Ephedrine	2		

[a] All drugs dissolved in saline were given by intraperitoneal injection.

The rise in analgetic response is expressed as the mean increase in time (seconds) required for the reflex tail twitch of a given group of rats before and after injection of the drug using the Wolff-Hardy-Goodell technique.

nervous system of these animals to the stimulant and depressant properties of the moieties embedded within the molecular structures.

It may be pointed out that in all such attempts to correlate chemical constitution with pharmacodynamic action the most desirable results are those which suggest an immediate translation of the chemical constitutional considerations into physiological terms. Thus, with the analgesics, consideration of "whole-molecule" properties gives little insight into the connection between their chemistry and pharmacology while recognition of physiologically active moieties within the whole structure gives a plausible interpretation of their actions with reference to the autonomic nervous system. Once these essential moieties are recognized one may approach the synthesis of new agents possessing the given activity with an almost infinite range of possible variations so long as the essential moiety relationships are maintained. Changes in the constitution of these molecules which increase or decrease their lipoid solubility, and penetrability into nervous tissue and cells will, of course, also effect quantitative changes in their activity. Further, the introduction of steric factors and groups which modify the electronic properties of the physiologically active moieties will cause quantitative changes in activity. Tentatively one might suggest that the ideal molecule for a given type of pharmacodynamic activity would be relatively inert with the exception of the essential moieties embedded in it and that any modifications that would be introduced should enhance the ease with which the molecule may reach its given site of action.

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The Quantitative Estimation of Sulfoxylate-Sulfur*

By C. K. BANKS, JACQUELINE D. MITULSKI, D. F. WALKER, and L. M. WHEELER

Existing analytical procedures for the determination of sulfoxylate-sulfur are inadequate for several compounds of pharmaceutical importance. Methods were developed for the determination and differentiation of this class of sulfur compounds.

THE increased use of sodium formaldehyde-sulfoxylate as a solubilizing agent for medicinal compounds containing aromatic amine groups has indicated the need for a method of estimating sulfoxylate-sulfur and related sulfur in such compounds. Not only is a method needed for the methylsulfoxylate linkage but also for its partially oxidized products which may not be identical with the methanesulfonate products, $-NHCH_2SO_3^-$.

Sodium formaldehyde sulfoxylate had been assayed with methylene blue in hot dilute acetic acid under nitrogen (1, 2), with ammoniacal copper sulfate (3–5) under hydrogen at 55°, and with iodine in "neutral" solution (2, 4). These

* Received July 22, 1948, from the Research Laboratories of Parke, Davis and Co., Detroit, Mich.

three procedures did not give equivalent results (2, 4). Attempts have also been made to analyze methylsulfoxylate derivatives of aromatic amines (1, 6–12). Elvove (8) suggested an indigotindisulfonic acid method for neoarsphenamine but Dyke and King (12) showed that the results obtained by this method were high. The methylene blue titration cited by Freedman (1) was found to yield values from 71 per cent to 90 per cent of the expected (12). Iodine has been used widely (6–12) but the severe criticism of this method by Freedman (1) and the data of Dyke and King (12) indicated that the results were not quantitative.

Since none of the methods as described appeared to be accurate, it was decided to investigate the iodine method. This choice was dictated by the larger general knowledge of iodimetry as well as avoidance of inert atmospheres and increased temperatures. Sodium formaldehyde sulfoxylate when titrated with iodine appears to react stoichiometrically according to Equation I, provided a few drops of acetic acid are added prior to titration. If the acetic acid is not added, the results may be somewhat irregular.

$$HOCH_2{\cdot}SO_2Na + 2I_2 + 2H_2O \xrightarrow{[H^+]} CH_2O + NaHSO_4 + 4HI \quad (I)$$

Titration of arylaminomethylsulfoxylates, however, was found to be incomplete, approximately 90 per cent of the stoichiometric amount of iodine being used as had been noted previously (12). Since a trace of acid appeared to be necessary for the titration of inorganic sulfoxylates, a slight amount of acetic acid was added prior to titration. This resulted in an increased consumption of iodine and the end point became more vague. When hydrochloric acid was used the titration became nearly quantitative but the end point was extremely unstable. Since the last part of the titration appeared to be due to a reaction having a slow velocity, the organic sulfoxylates were dissolved in water, acidified with acetic acid and then added to an excess of iodine in an iodine flask, acidified with hydrochloric acid and allowed to stand for varying periods of time. Back-titration with thiosulfate indicated that the maximum amount of iodine was used after forty minutes and the value was unchanged up to several hours. Furthermore, the quantity of iodine used was stoichiometric according to Equation II.

$$RNHCH_2{\cdot}SO_2Na + 2I_2 + 3H_2O \xrightarrow{[H^+]} RNHCH_2OH + NaHSO_4 + 4HI \quad (II)$$

The procedure was verified on a sample of known purity. This procedure was then used on about forty different samples, representing a number of arylaminomethylsulfoxylates. A total of about seventy-five duplicate assays were made. The statistical precision of the method was determined by the variations of the individual assays from their average in parts per thousand.

Experiments with arylaminomethanesulfonates indicated no absorption of iodine. While the procedure indicated a small variable blank, no blank correction was made except on hot days when the maintenance of standard solutions was difficult. The thiosulfate equivalence of the iodine was determined for each group of assays. A definite excess of iodine had to be present to give duplicable results.

Since arylaminomethanesulfoxylates are extremely susceptible to air oxidation, sulfoxylate oxidation products were also titrated. Some iodine was utilized by these compounds, raising the question as to whether this was due to an intermediate compound or to a mixture of oxidized and unoxidized compounds. The literature on this subject is conflicting. It was noted that Heyl and Greer (2) had claimed that certain lots of sodium formaldehydesulfoxylate, after titration with iodine in unbuffered solution, had absorbed a further quantity of iodine in bicarbonate buffers. A similar variation was noted with our arylaminomethylsulfoxylates. This end point was extremely variable. When the samples, however, were added to excess iodine and made sufficiently alkaline to bleach the solution, and then allowed to stand an hour, acidification returned any unused iodine and the stoichiometry appeared to fit Equations III and IV.

$$HOCH_2{\cdot}SO_2Na + 3I_2 + 3H_2O \xrightarrow{[OH^-]} HCOOH + NaHSO_4 + 6HI \quad (III)$$

$$RNHCH_2{\cdot}SO_2Na + 3I_2 + 3H_2O \xrightarrow{[OH^-]} HCONHR + NaHSO_4 + 6HI \quad (IV)$$

Arylaminomethanesulfonates, when treated similarly, absorbed no iodine. Oxidation products of arylaminomethylsulfoxylates also reacted with alkaline iodine, utilizing a different quantity of iodine under alkaline conditions from that used in acids.

EXPERIMENTAL

Standard solutions were prepared according to Kolthoff and Sandell (13). Iodine, ca. 0.05 *N*, was checked for possible iodate contamination before each use (14). Sodium thiosulfate, ca. 0.05 *N*, was standardized each day.

Acid Sulfoxylate Determinations.—Accurately weigh samples of the compound to be assayed so as to have not more than 7 to 8 mg. of sulfoxylate-sulfur per sample. Accurately transfer 30-ml. quantities of standard iodine (ca. 0.05 *N*) to iodine flasks. Dissolve the samples in distilled water (ca. 10 ml.) using a drop of *N* sodium hydroxide if necessary. Acidify the samples with 2 ml. of glacial acetic acid (methylenesulfoxylates and methylsulfonates do not precipitate at this point) and transfer quantitatively to the iodine flasks. Add 3 ml. of concentrated hydrochloric acid, stopper, mix thoroughly and allow to stand an hour at room temperature. Titrate the excess iodine with standard sodium thiosulfate (ca. 0.05 *N*) using starch solution at the end point. Each ml. of 0.05 *N* iodine is equivalent to 0.0004004 Gm. of sulfoxylate sulfur. The complete calculation is given by:

$$\%S \text{ (sulfoxylate)} = \frac{[\text{Ml. } (I_2) \times N(I_2) - \text{ml. } (S_2O_3^{=}) \times N(S_2O_3^{=})] \times 0.03206}{\text{Weight of sample} \times 4}$$

If 0.1 *N* solutions are used, the sample may contain up to 15 mg. of sulfoxylate sulfur.

Alkaline Sulfoxylate Determinations.—Accurately weigh samples of the compound to be assayed so as to have not more than 8 mg. of sulfoxylate sulfur per sample. Accurately transfer 50 ml. of standard iodine (ca. 0.05 *N*) to iodine flasks. Dissolve the samples as in the acid determination and transfer quantitatively to the iodine flasks. Add 0.1 *N* sodium hydroxide until the iodine color is bleached. Allow to stand an hour at room temperature, acidify with an equivalent of concentrated hydrochloric acid and 3 ml. excess, allow to stand fifteen minutes and titrate the excess iodine with standard sodium thiosulfate solution (ca. 0.05 *N*) using starch indicator. Each ml. of 0.05 *N* iodine is equivalent to 0.0002672 Gm. of sulfoxylate sulfur. The complete calculation is given by:

$$\%S \text{ (sulfoxylate)} = \frac{[\text{Ml. } (I_2) \times N(I_2) - \text{ml. } (S_2O_3^{=}) \times N(S_2O_3^{=})] \times 0.03206}{\text{Weight of sample} \times 6}$$

Disodium Dimethylenesulfoxylate of 4,4′-Diaminodiphenylsulfone.—A sample of the disulfoxylate derivative of 4,4′-diaminodiphenylsulfone of known purity was assayed as described. The results are shown in Table I.

Anal.—Calcd. for $C_{14}H_{14}O_6N_2S_3Na_2$: S, 14.32%. Found: S (sulfoxylate-acid) 14.30% (av. 8); (sulfoxylate-alkaline) 14.26% (av. 6).

TABLE I.—ESTIMATION OF SULFOXYLATE SULFUR IN THE DISODIUM DIMETHYLENESULFOXYLATE OF 4,4′-DIAMINODIPHENYLSULFONE

Per Cent Sulfoxylate Sulfur	
Acid Detn.	Alkaline Detn.
14.20	14.32
14.31	14.44
14.12	14.20
14.42	14.17
14.28	14.28
14.34	14.16
14.38	...
14.27	...

Sodium Formaldehydesulfoxylate.—Samples of reagent grade sodium formaldehydesulfoxylate (Merck) were assayed by the acid and alkaline methods and by direct titration in the presence of acetic acid. The results are shown in Table II.

TABLE II.—ESTIMATION OF SULFOXYLATE SULFUR IN SODIUM FORMALDEHYDESULFOXYLATE

Per Cent Sodium Formaldehydesulfoxylate		
Acid Detn.	Alkaline Detn.	Direct Iodine Detn.
99.76	99.53	99.67
99.75	99.30	99.77
99.89	99.71	99.44
99.90	99.77	99.80
Averages		
99.83	99.58	99.67

While the above averages are not conclusive checks, the allowable variation in the methods as shown below indicates the above values as obtained by the acid and alkaline methods to be not significantly different from the direct titration value.

Deviation of Assays.—A group of 77 assays on 20 samples was listed by deviation from the averages in parts per thousand. The data when grouped in unit parts per thousand were analyzed statistically. (Table III.)

TABLE III.—STATISTICAL ANALYSIS OF ASSAY DEVIATIONS

Deviation, P. P. T.	Frequency	$N(d')^2$
0–1	15	3.75
1–2	10	22.50
2–3	12	75.00
3–4	11	134.75
4–5	8	162.00
5–6	7	211.75
6–7	5	211.25
7–8	4	225.00
8–9	2	144.50
9–10	2	180.50
10–11	1	110.25
Total	77	1481.25
Variance		19.4901
Standard Deviation (single assay)		4.4
Standard Deviation (two assays)		3.1
Standard Deviation (four assays)		2.2
99% limits (single value)		11.5 p. p. t.
99% limits (av. two)		8.2 p. p. t.
99% limits (av. four)		5.8 p. p. t.

SUMMARY

An assay method for determining the per cent sulfoxylate sulfur in arylaminomethylsulfoxylates involving acidic iodine oxidation has been presented. The precision of single assay values by the method is 11.5 parts per thousand at 99 per cent probability. While the precision is not exceptional, the accuracy of multiple determinations would appear to be of the order of 6 parts per

thousand. A check assay for methylenesulfoxylate linkages has also been presented, utilizing both acid and alkaline oxidation by iodine.

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Hydrogenolysis as a Method for Determining Chlorguanide (Paludrine)*

By ROBERT C. SHULTZ

Chlorguanide may be determined quantitatively by hydrogenolysis in acid solution with zinc amalgam to form a primary aromatic amine which is subsequently measured colorimetrically by application of a modified Bratton-Marshall reaction.

THROUGH the use of zinc amalgam in an acid medium and an efficient shaking machine, chlorguanide, N_1-chlorophenyl-N_5-isopropyl biguanide, can be cleaved quantitatively to form a primary aromatic amine, probably *p*-chloroaniline or aniline. The amine can be measured conveniently by diazotization followed by coupling to form an azo dye according to a conventional modification of the original Bratton-Marshall reaction (1). Spinks and Tottey describe a method for the determination of chlorguanide in blood and tissue in which *p*-chloroaniline is formed by the hydrolysis of chlorguanide in an autoclave for a period of four hours (2, 3). In this procedure the sample must be sealed in an ampul. Zinc and zinc amalgam have been used to split pteroylglutamic acid by a similar procedure described by Hutchings, *et al.* (4).

EXPERIMENTAL

A solution of a commercial sample of chlorguanide in 0.5 *N* hydrochloric acid (supplied by E. I. du Pont de Nemours and Company, Inc.) was analyzed by the procedure described in the present communication, using concentrations of from 1 to 6 mg. in each 10 ml. of solution. A linear relationship between concentration and color production was found (Fig. 1). In order to determine the optimal time required for complete cleavage, the amine formed was measured at ten-minute intervals during an eighty-minute period of shaking with zinc amalgam. No further increase could be detected after sixty minutes (Fig. 2).

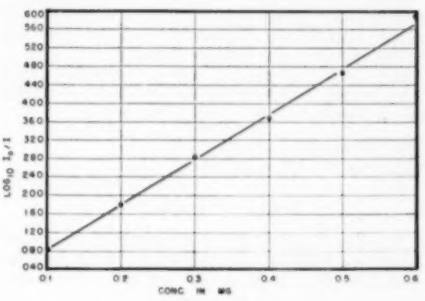

Figure 1.

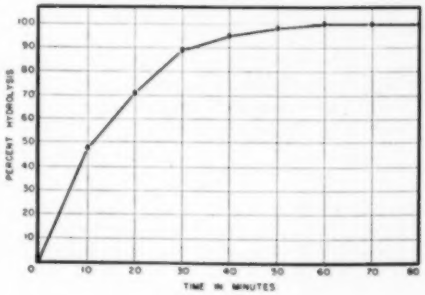

Figure 2.

* Received August 11, 1948, from the Pharmaceutical Research Laboratories, Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

For the work in this laboratory a "Precision" Equipoise heavy-duty shaker No. 5855 was employed. Some variation in the time required for quantitative cleavage may result, depending on the relative efficiency of the shaker used. The procedure, as developed particularly for application to drug products, follows.

Reagents—Zinc amalgam. To 20 ml. of mercury add 10 Gm. of mossy zinc. Heat with stirring to 150° until the zinc is dissolved.

0.1% aqueous sodium nitrite.

0.5% aqueous ammonium sulfamate.

0.1% freshly prepared aqueous *N*-1(1-naphthyl) ethylene diamine dihydrochloride.

0.5 *N* hydrochloric acid.

Chlorguanide standard solution: 4 mg. per 100 ml. in 0.5 *N* HCl.

Procedure.—Prepare an extractive or solution of the sample and adjust it to contain approximately 0.4 mg. of chlorguanide in each 10 ml. of 0.5 *N* hydrochloric acid. Transfer 50–60 ml. of this solution to a bottle, add 5 ml. of the zinc amalgam and stopper tightly. Shake mechanically for one hour. Transfer exactly 10 ml. of the solution reduced in this way to a 100-ml. volumetric flask; add 5 ml. of *N* HCl and 35 ml. of H_2O. Add as directed: 5.0 ml. of 0.1% sodium nitrite—wait two minutes; 5.0 ml. of 0.5% ammonium sulfamate—wait three minutes; and 5.0 ml. of 0.1% *N*-(1-naphthyl) ethylene diamine dihydrochloride. Wait ten minutes and dilute to 100 ml. with 0.15 *N* hydrochloric acid. Mix well and measure the transmission in a Beckman spectrophotometer at a wave length of 550 mμ, or in a suitable colorimeter. Calculate the extinction ($\log_{10} I_0/I$) and compare it with that found for a 10-ml. portion of the standard chlorguanide solution run in the same way and at the same time.

SUMMARY

A method is described wherein chlorguanide is determined through cleavage by hydrogenolysis in acid solution with zinc amalgam to yield a primary aromatic amine which is subsequently measured colorimetrically through the application of a modified Bratton-Marshall reaction.

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Microtoxicology. IV. The Identification of Antihistaminic Drugs of the Thenyl Series*

By THOMAS J. HALEY† and GEORGE L. KEENAN‡,§

The optical crystallographic properties of antihistaminic drugs of the thenyl series have been determined and are recorded in this report. Other means of identification and differentiation involving the use of six common colorimetric alkaloidal reagents are described. The crystallographic properties described offer the best means of identification.

THE extensive use of such drugs as Benadryl Pyribenzamine in the treatment of allergenic conditions has led to the synthesis and therapeutic use of an increasing number of antihistaminic agents. Among the latest drugs of this group are Thenylene or Histadyl (N,N-dimethyl-N′-(2-pyridyl)-N′-(2-thenyl)-ethylenediamine) and its halogenated derivatives Chlorothen (N,N - dimethyl - N′ - (2 - pyridyl) - N′ - (5-chloro - 2 - thenyl) ethylene diamine) and Bromothen (N,N-dimethyl-N′-(2-pyridyl)-N′-(5-bromo-2-thenyl) ethylenediamine)(1, 3, 13). Table I lists the physical constants for these compounds.

TABLE I.—PHYSICAL CONSTANTS OF THE THENYL ANTIHISTAMINES

Compound	Boiling Point °C.	Mm.	Melting Point, °C.	Reference
Histadyl base	173–175	3	.....	14
Thenylene HCl	.....	..	161–162	..
Methoiodide	.....	..	156–157 dec.	..
Chlorothen				
Base	155–156	1	.....	1
HCl	.....	..	106–108	..
(Tagathen)				
Citrate	.....	..	116–118	..
Bromothen				
Base	173–175	1	.....	1
HCl	.....	..	124–126	..

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§ The generous supplies of Histadyl (Thenylene) were furnished by Eli Lilly and Co. and Abbott Laboratories, and the Chlorothen and Bromothen by Lederle Laboratories.

The pharmacological activity (2, 7, 8, 10) and therapeutic effects (4,9) of these compounds have been described but as yet no simple means for their identification has been published. This investigation was undertaken to determine the reactivity of these Thenyl antihistaminics with several simple alkaloidal precipitants and color reactions to see if such tests could serve as a means of identification. Further, comparison was made with the color reactions already recorded in the literature to prevent confusion and misinterpretation of the results obtained.

EXPERIMENTAL

The reagents were prepared as directed by Thienes and Haley (12). The compounds were tested by placing one drop of a reagent on a microscope slide and adding about one milligram of the drug to it. The results of this addition were observed for about thirty minutes and changes taking place were recorded. In the case of the acidic colorimetric reagents the end point was a charring of the drug, but only the colors appearing before this were used for identification. Table II gives the results of precipitation and colorimetric tests.

Inasmuch as the precipitation and colorimetric tests were almost identical for each of the Thenyl compounds, the optical crystallographic properties of pure compounds were studied.

Histadyl.—In ordinary light, the habit is rather thick plates, hexagonal in outline, and readily breaking up into irregular fragments.

Refractive indices: $\alpha = 1.588$, $\beta = 1.654$, $\gamma = >1.695$, but <1.734; all ± 0.002. α and β are most frequently observed.

In parallel polarized light (crossed nicols): Many of the fragments do not extinguish sharply with crossed nicols.

In convergent polarized light (crossed nicols): Partial biaxial interference figures frequently shown with one optic axis in the microscopic field. Optic sign: (−).

Chlorothen Hydrochloride.—In ordinary light, the substance consists of massive fragments, some of which are rectangular in shape.

Refractive indices: $\alpha = 1.553$ (commonly shown), $\beta = 1.625$ (not common), $\gamma = >1.734$; both ± 0.002.

In parallel polarized light (crossed nicols): Many fragments do not extinguish sharply with crossed nicols.

In convergent polarized light (crossed nicols): Optic axis figures occasionally shown, and frequently inclined to the axis.

Chlorothen Citrate.—The substance occurs as very minute fragments, in ordinary light, consisting of plates and shreds. The plates frequently are rhombic in outline, and the shreds consist of plates tipped on edge.

Refractive indices: $\alpha = 1.583$, $\beta = 1.603$, $\gamma = 1.645$; all ± 0.002. β and γ frequently shown on plates on edge.

In parallel polarized light (crossed nicols): Many of the fragments do not extinguish sharply with crossed nicols.

In convergent polarized light (crossed nicols): Optic axis figures can be observed, but they are the exception rather than the rule, due to the small size of the plates.

Bromothen Hydrochloride.—In ordinary light, the substance is rod-like in habit, the rods being very small.

Refractive indices: $\alpha = 1.617$ (common), $\beta = 1.654$, $\gamma = 1.734$ (also common); all ± 0.002.

In parallel polarized light (crossed nicols): The extinction usually is inclined on the rods and the sign of elongation is positive. The rods, however, do not extinguish sharply.

In convergent polarized light (crossed nicols): Partial biaxial interference figures showing the optic axis inclined occasionally are shown, but rods are too small to show ideal figures.

The photomicrographs of the pure compounds were taken with a Visicam photographic attachment.

TABLE II.—REACTION OF THENYL ANTIHISTAMINES WITH VARIOUS REAGENTS

Reagent	Histadyl HCl	Bromothen HCl	Chlorothen HCl	Tagathen Chlorothen Citrate
Conc. H_2SO_4	Burnt orange changing to blood red, finally deep purple	Magenta, then deep purple	Magenta, then deep purple	Magenta, then deep purple
Conc. HNO_3	Purple-pink changing to brown	Orange, changing to lemon yellow	Yellow	Orange-red
Mandelin's	Burnt orange	Deep reddish orange	Deep reddish orange	Deep reddish orange
Marquis	Orange-brown changing to purplish pink	Bright red	Carmine red with a slight purple cast	Brilliant magenta
Fröhde's	Dark brown with black streaks	Deep reddish purple	Deep reddish purple	Deep reddish purple
Buckingham's	Brown, then black	Deep reddish purple	Deep reddish purple	Deep reddish purple
Chloroplatinic acid	Branched bundles of rods in feather-like agglomerates	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate
Chlorauric acid	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate
Picric acid saturated aqueous soln.	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate	Amorphous precipitate

Fig. 1.—Thenylene. Magnification 115×.

on a Band L binocular microscope using the 16-mm. objective and the 10× eyepiece. All of the compounds have distinctive crystalline structure which may be used as a further means for their identification and differentification. See Figs. 1, 2, 3, and 4 for the photomicrographs.

DISCUSSION

Although papaverine, narceine, narcotine, physostigmine, codeine, morphine, strychnine, thebaine, and veratrine give various shades of yellow, yellow-red, or orange with nitric acid (11), the color reactions of these alkaloids with concentrated sulfuric acid (11) are so different from those of the Thenyl antihistamines that no difficulty would be encountered in their differentiation. With Mandelin's reagent (11), cinchonine, cocaine, pilocarpine, quinine, solanine, thebaine, and veratrine produce colors similar to those given by the Thenyl compounds, but here again comparison of their reactions to other colorimetric reagents would differentiate the compounds from each other. The colors produced with Marquis' reagent (11) are different from any of those recorded in the literature and thus this reagent is the best one for differentiation and identification of Thenyl compounds not only from each other but from other drugs and alkaloids. Both aconitine and veratrine give a brownish color with Buckingham's reagent (11) but the final color is considerably different from the color obtained with Histadyl. The other Thenyl compounds give the same colorimetric reaction, thus preventing their differentiation with this reagent. However, the color does differ from any previously reported for other compounds so that the reaction is of value in

Fig. 2.—Chlorothen. Magnification 75×.

differentiating these antihistaminics from other compounds or alkaloids. Aconitine, berberine, brucine, colcynthin, digitalin, morphine, narceine, and solanine give varying degrees of brownish coloration with Fröhde's reagent (11) but all of these alkaloids may be differentiated from Histadyl by their preliminary or final colors. The other Thenyl compounds cannot be differentiated from each other by this test but can be differentiated from brucine, colcynthin, curarine, digitalin, morphine, nicotine, papaverine, thebaine, or veratrine. These latter compounds show changing colors while the

Fig. 3.—Tagathen. Magnification 265×.

Fig. 4.—Bromothen. Magnification 65×.

Thenyl antihistaminics develop only one stable color. Benadryl and Pyribenzamine both produce colors with the color reagents used with Thenyl compounds, but the color reactions are so different that no confusion would result when comparisons are made (5).

Keenan (6) has described definite crystalline precipitates for both Benadryl and Pyribenzamine with chloroplatinic acid but none with chlorauric acid. The crystalline precipitate formed when Histadyl is mixed with chloroplatinic acid differs greatly from those formed by both Benadryl and Pyribenzamine. The other Thenyl antihistaminics could be identified by their reactions with any of the precipitants used because amorphous precipitates were formed in all cases.

SUMMARY

A means for the identification and differentiation of the Thenyl antihistaminic drugs has been described. The six common alkaloidal colorimetric reagents used gave better results than three precipitation reagents. However, the optical crystallographic properties described offer the best means for the identification of the Thenyl antihistaminics.

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The Bacteriological Aspects of Deionized Water*

By PHILIP C. EISMAN, F. C. KULL, and R. L. MAYER

A bacteriological study was made of demineralizing units containing synthetic anion and cation exchange resins for the production of water with low mineral content. The conditions studied were: the chemical purity of the effluent, intervals of time elapsing between successive operations of the deionizing units, and the frequency of resin regeneration. The results obtained indicate that there exists a wide variation in the bacterial content of deionized water which is conditioned by the manner in which these units are operated and that the bacterial content of the effluent bears no relationship to its chemical purity.

THE DISTILLED water ordinarily used in chemical and bacteriological laboratories and in the pharmaceutical, food, and related industries is being replaced to a considerable extent by the so-called demineralized or deionized water prepared by the action of synthetic ionic exchange resins. Because of the nature of these resins, deionizing units are also being employed in a variety of operations such as the removal of ionized impurities from solutions of nonelectrolytes (1) and for the separation and purification of ionizable biologicals such as vitamins (2) and amino acids (3).

Deionized water, when properly prepared, corresponds to distilled water with respect to the absence of inorganic salts (4). Moreover Harrisson and co-workers (5) have reported that resinous exchanger systems would produce water equal in quality to that required by U. S. P. specifications and that the pyrogen content was unaffected by the exchange process. But contrary to distilled water, it may contain significant amounts of organic matter, depending upon the nature of the raw water entering the demineralizing unit (2). Furthermore, distilled water by the very nature of its preparation is sterile at the time of its production and only the conditions of its storage and handling determine the nature and extent of its subsequent bacterial population. Observations made in our laboratory indicated that, unlike distilled water, deionized water was only occasionally sterile and often grossly contaminated at the time of production. Since the use of deionized water in various laboratories and

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manufacturing processes makes it necessary that it be relatively free of microorganisms, a bacteriological study of this type of water was undertaken.

Since many desirable features are associated with the use of demineralizing units, it may be expected that a more general, industry-wide acceptance of this process will take place in the near future. Experimental data yielding information regarding the bacterial aspects of the water so produced are therefore not only of timely interest, but would indicate those conditions of operation yielding a product of low bacterial content.

In the deionizing units such as that constructed by the American Cyanamid Company (Filt-R-Stil Demineralizers) and which have furnished the water investigated during the course of the present work, raw water is subjected to the successive action of synthetic cation and anion resins and is as a result almost completely demineralized. As shown in Fig. 1, which is adapted from data obtained from the American Cyanamid Company, each of the units consists of four resin beds arranged in series and alternately containing cation and anion synthetic resins. The end product or effluent, which has a *p*H value usually between 6.5 and 7.5, contains less than 2 parts per million of ionizable salt (less than the 2 mg. of salt residue per liter of water) and is therefore chemically as pure as properly prepared distilled water in so far as ionizable inorganic residues are concerned.

The amount of raw water a deionizing unit will efficiently process depends upon the ionizable salt content of the water. A Barnstead purity meter expressing values in terms of ionizable salt content, indicates the actual efficiency of the unit at any given moment. Supplementing the purity meter is a system of green and red indicator light bulbs which operate according to the electrical conductivity of the water issuing from the unit. A green light indicates an effluent containing a mineral content below a predetermined maximum, while a red light denotes an excessive mineral content. When the capacity of the resins to remove ions has been exhausted as shown by the purity meter or the red light, it is necessary to halt the deionizing process and regenerate both the cation and anion exchange resins. The frequency between two successive regenerations is dependent upon the volume

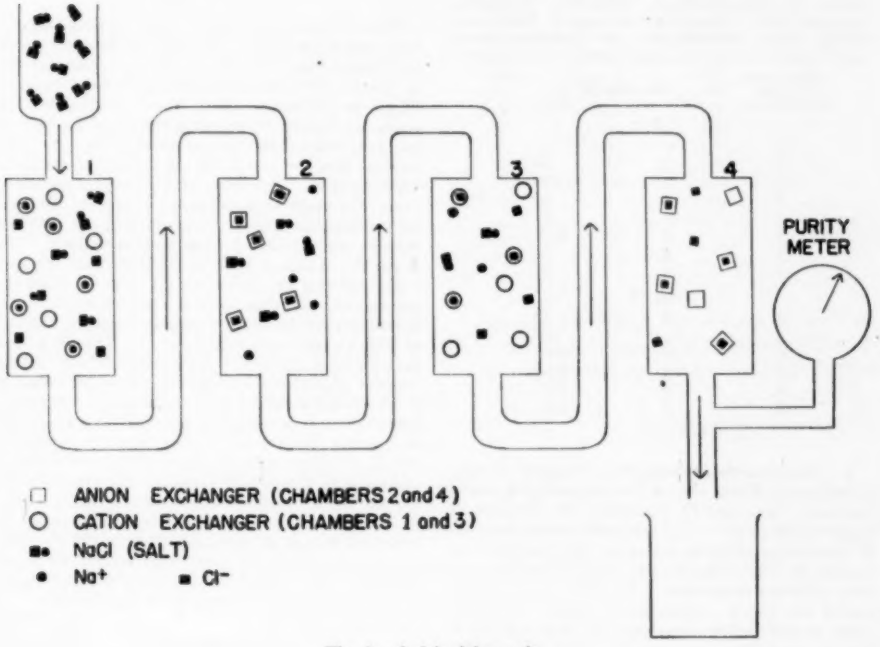

Fig. 1.—A deionizing unit.

of water processed and is also conditioned by the amount of dissolved mineral matter contained in it. It is therefore possible that a unit with a capacity for treating 1500 gallons may require regeneration only after several weeks of use when relatively small amounts of water with a low original mineral content are occasionally required, or it may become necessary after two or three days if larger amounts are passed through.

EXPERIMENTAL

1. Determination of the Numbers of Organisms Contained in Freshly Prepared and Chemically Suitable Effluent at Intervals during Its Production.—One-milliliter amounts of effluent samples collected from 2 different units at random times during a period of forty-two days were plated for bacterial count. All agar plates were incubated for seventy-two hours at 37°. The results (Table I) showed a considerable degree of variation in the bacterial content of the various samples and indicated that the numbers of organisms in the effluent bore a direct relationship to the time elapsing between successive regenerations.

TABLE I.—RELATIONSHIP BETWEEN BACTERIAL CONTENT OF CHEMICALLY SUITABLE DEIONIZED WATER AND FREQUENCY OF REGENERATIONS

Days After Previous Regeneration	Bacteria/Ml. in Influent	Effluent
	Unit I	
7	0	1
1	0	0
2	1	32
1	4	0
3	0	0
7	0	150
2	4	0
8	3	5
	Unit II	
1	5	1
8	14	16
16	0	520
22	0	110
34	1	700
42	2	125

2. Relationship between the Chemical Quality of Deionized Water and Its Corresponding Bacterial Content.—Immediately following the process of regeneration of the resins and prior to the issuance of chemically suitable water as indicated by the system of colored lights, 100-ml. samples of water were collected aseptically at frequent intervals and plated for count. Additional samples were also taken during periods ranging from twelve hours to twenty days after regeneration. For this study, the same two deionizing units as used in the preceding experiment were available, one of which (Unit I), being used almost continuously, was regularly regenerated at intervals of no greater than three or four days, while the other (Unit II), operated infrequently, was regenerated approximately every twenty days. From the results obtained (Tables II and III) it becomes apparent that the bacterial content of the effluent water bears no relationship to its chemical quality.

A very important difference between the two units is readily apparent. In Unit I, which is regenerated every three or four days, there is but little growth of bacteria since the product is practically sterile in spite of a count of 60 bacteria per ml. in the raw water. On the other hand, in Unit II, which requires infrequent regenerations, there is considerable growth and release of bacteria. Actually, this unit produces water containing more than 100 times as many organisms per ml. as are contained in the pretreated water, and this in spite of the fact that the product is chemically pure.

3. Types of Bacteria Found in Deionized Water.—Since relatively large numbers of bacteria were encountered in the water issuing from the demineralizing units, a survey was made to determine the types present. The predominating organisms found in freshly prepared deionized water were pseudomonas types normally present in fresh water. The remaining forms were Gram-negative, nonmotile, or motile rods. Spore-forming bacilli were not generally observed. From these findings it would seem that the synthetic resins do not alter the flora of the water passing through them.

DISCUSSION

The data presented in this report clearly indicate that deionized water differs from distilled water with respect to its bacterial content. The presence or absence of bacteria depends upon the frequency of use of the units. After fresh regeneration, the resins are capable of removing the small numbers of bacilli contained in the raw water during the subsequent first few hours and the product is sterile or very nearly so. However, with infrequent regenerations, the resin beds represent a culture medium for retained bacteria which are flushed out with the effluent when the unit is placed into operation. This is readily apparent when it is shown that the raw water entering the unit contains from 40 to 90 organisms per ml. while the resulting deionized water, although chemically acceptable, contains upward of 6000 to 12,000 organisms per ml. Indeed, as has been found in our studies, subsequent storage of this product containing, in addition to bacilli, organic matter not removed by the resins may result in a bacterial content exceeding a million organisms per ml. within twenty-four hours after preparation. The implications with respect to the introduction of pyrogens as a result of excessive bacterial growth are worthy of serious consideration. On the other hand, it is possible to produce demineralized water relatively free of microorganisms provided that the units are used under conditions similar to those employed with Unit I; that is, daily use and regeneration of the resins every third or fourth day.

SUMMARY

A bacteriological study has been made of demineralizing units employing synthetic cation and anion exchange resins for the production of water with low mineral content. It has been established that there exists a wide variation

TABLE II.—CORRELATION BETWEEN BACTERIAL NUMBERS AND CHEMICAL PURITY OF DEIONIZED WATER—UNIT I

Time of Sampling, Min.	Freshly Regenerated		12 Hours after Regeneration		24 Hours of Regeneration	
	Bacteria Per Ml.	Chemical Quality[a]	Bacteria Per Ml.	Chemical Quality	Bacteria Per Ml.	Chemical Quality
Start	0	Poor	0	Poor	210	Poor
5	1	Poor	0	Good	0	Poor
10	0	Poor	0	Good	0	Good
15	0	Poor	0	Good	2	Good
20	0	Poor	0	Good	0	Good
30	5	Poor	2	Good	4	Good
40	0	Poor	0	Good	0	Good
50	0	Poor	1	Good	0	Good
60	1	Poor	0	Good	0	Good
70	0	Good	0	Good	2	Good
80	0	Good	0	Good	0	Good
90	0	Good	0	Good	1	Good
120	0	Good	0[b]	Good	0[c]	Good
Raw water supply	60		40		34	

[a] As indicated by the electrical conductivity of the water.
[b] Total output: 150 gallons.
[c] Total output: 300 gallons.

TABLE III.—CORRELATION BETWEEN BACTERIAL NUMBERS AND CHEMICAL PURITY OF DEIONIZED WATER—UNIT II

Time of Sampling, Min.	Freshly Regenerated		144 Hours after Regeneration		20 Days after Regeneration	
	Bacteria Per Ml.	Chemical Purity	Bacteria Per Ml.	Chemical Purity	Bacteria Per Ml.	Chemical Purity
Start	0	Poor	6500	Poor	12,000	Poor
5	0	Poor	5220	Poor	3,600	Poor
10	1	Poor	800	Poor	4,900	Poor
15	1	Poor	540	Poor	5,100	Poor
20	2	Poor	300	Good	6,100	Good
30	10	Poor	450	Good	2,200	Good
40	21	Poor	660	Good	890[b]	Good
50	27	Poor	1000	Good	360	Poor
60	17	Good	1440	Good	390	Poor
70	20	Good	1220	Good	550	Poor
80	22	Good	700	Good	1,020	Poor
90	26	Good	150[a]	Good	1,000	Poor
Raw water supply	300		40		90	

[a] Total output: 175 gallons.
[b] Total output: 645 gallons.

in bacterial content in deionized water which is conditioned by the manner in which these units are operated. An almost sterile product may be produced provided the deionizing units are used daily and the resins regenerated every three or four days. If, on the other hand, the resins are regenerated at infrequent periods, that is, several days or weeks apart, the bacterial content of the effluent, notwithstanding its chemical purity, may be excessive, being considerably greater than the numbers of organisms in the raw water entering the unit. The storage of such water gives rise to exceedingly large numbers of bacteria within twenty-four hours after collection.

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The Solubility of Theophylline in Aqueous Amine Solutions*

By E. E. LEUALLEN† and ARTHUR OSOL‡

A study has been made of the solubility of theophylline in aqueous solutions of certain amines. The molar solubility of theophylline has been plotted against molar concentrations of monomethylamine, dimethylamine, trimethylamine, monoethylamine, diethylamine, mono-*n*-propylamine, di-*n*-propylamine, mono-*n*-butylamine, and di-*n*-butylamine. The solubilizing effect decreases with increasing length of the carbon chain; thus monomethylamine has an effect greater than that of any other amine investigated. Monomethylamine has greater solubilizing effect than does dimethylamine, which in turn is more effective than trimethylamine. The solubility increases with increasing concentration of primary amine until a point of maximum solubility is reached, thereafter solubility decreases with increasing amine concentration. The maximum solubility increases with increasing length of the carbon chain.

MANY substances have been shown to increase the water solubility of theophylline, with the great majority of these substances being amines. In some instances double compounds have been isolated and a few of these have become important therapeutic commodities. In 1907 a German patent (1) was issued for the preparation of such a compound by reaction of theophylline and piperazine and subsequent evaporation *in vacuo* to obtain a solid product. Additions to this patent covered the reaction of barium theophylline and piperazine sulfate (2), the reaction of theophylline and such amines as ethylenediamine, hexamethylenediamine and lysidine (3), and the fact that these amines produced soluble double compounds in less than molar ratios (3,4). The first United States patent (5) for this type of product was issued in 1909 to the same organization. This patent discloses that theophylline forms water soluble compounds with primary and secondary diamines and indicates the use of ethylenediamine, pentamethylenediamine, and hexamethylenediamine.

Among other substances which have been shown to be solubilizing agents for theophylline are monoethanolamine (6), diethanolamine (7), triethanolamine (6), chlorophylline (8), monoamino polyhydric alcohols such as glucamine, methyl glucamine, ethyl glucamine, dimethyl glucamine and ethanol glucamine (9, 10), mono-, di- and tri-isopropanolamine (11), β-phenylisopropylamine and its derivatives (12) and 2-diethylaminoethanol (13). Greenbaum has stated (14) that the compounds formed from theophylline and either morpholine, propylenediamine, ethylamine, piperidine or triethylenetriamine, in monomolecular and polymolecular proportions, were not sufficiently soluble in water to be therapeutically useful. Complex compounds of theophylline and salts of *ortho-* or *meta*-hydroxybenzoic acid have been described in several patents (15–19). Compounds or molecular associations have also been reported to be formed with dehydrocholic acid (20), phenobarbital (21–23), 3-chloro-1,2-dihydroxypropane (24), and papaverine (25). A product identified as theophylline aminoisobutanol has been studied experimentally and clinically (26–28).

In the present study a determination has been made of the solubility of theophylline in aqueous solutions of the following aliphatic amines: monomethylamine, dimethylamine, trimethylamine, monoethylamine, diethylamine, mono-*n*-propylamine, di-*n*-propylamine, mono-*n*-butylamine and di-*n*-butylamine. Several concentrations of each amine have been prepared in order to observe the effect of increasing concentration on solubilizing ability. Findings are reported in Tables I to IX.

EXPERIMENTAL

1. Materials.—The theophylline used in this study was a U. S. P. product, recrystallized from water and dried at 100°. The amines were commercial grade supplied by Eastman Kodak in aqueous solution. Dilutions were made with freshly distilled water.

2. Method.—In each determination an excess of theophylline was added to the amine dilution contained in a 200-cc. hard glass bottle and a paraffined cork inserted and tied in place. The bottles were rotated in a constant temperature bath containing water at 25° ± 0.02° for not less than forty-eight hours. The excess solute was permitted to

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TABLE I.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONOMETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.5364	0.8399
0.7903	1.200
1.028	1.520
1.244	1.783
1.696	2.293
1.802	2.409
1.809	2.376
1.991	1.848
2.106	1.780
2.691	1.594
3.538	1.414
3.779	1.376

TABLE II.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DIMETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.3081	0.5140
0.6355	0.9850
1.008	1.470
1.238	1.743
1.695	2.235
1.765	2.302
2.356	2.868
2.955	3.259

TABLE III.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF TRIMETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.2525	0.3905
0.5164	0.7367
0.8411	1.093
1.078	1.376
1.532	1.743
1.930	2.060
2.418	2.366

TABLE IV.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONOETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.4982	0.7818
1.021	1.480
1.750	2.305
1.812	2.357
2.327	2.869
2.406	2.677
2.437	2.666
2.806	2.397

TABLE V.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DIETHYLAMINE

Molarity of Amine	Molarity of Theophylline
0.4723	0.7462
1.066	1.503
1.335	1.813
1.379	1.864
2.170	2.558
2.669	2.916

TABLE VI.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONO-*n*-PROPYLAMINE

Molarity of Amine	Molarity of Theophylline
0.7020	1.080
1.115	1.620
2.458	2.913
2.605	2.895
2.918	2.730
3.321	2.553
3.687	2.446

TABLE VII.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DI-*n*-PROPYLAMINE

Molarity of Amine	Molarity of Theophylline
0.4398	0.6965
0.7925	1.163
1.281	1.680
1.356	1.735
1.409	1.804
1.556	1.945
2.207	2.399
2.243	2.436

TABLE VIII.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF MONO-*n*-BUTYLAMINE

Molarity of Amine	Molarity of Theophylline
0.6063	0.9379
1.154	1.629
2.481	2.863
2.499	2.886
2.721	3.071
2.811	3.087
2.886	3.030
4.132	2.605
4.491	2.489

TABLE IX.—SOLUBILITY OF THEOPHYLLINE IN SOLUTIONS OF DI-*n*-BUTYLAMINE

Molarity of Amine	Molarity of Theophylline
0.3396	0.5580
0.5379	0.8190
0.6707	0.9954
1.238	1.636
1.822	2.110
1.940	2.196
2.125	2.278
2.212	2.320
2.490	2.460
2.648	2.496
2.694	2.502

settle and samples of the supernatant saturated solution were withdrawn by means of a dry 10-cc. calibrated pipette, to the lower orifice of which was fastened by means of rubber tubing a 5-cm. length of glass tubing having a drawn end to permit easy attachment and glass-to-glass contact; cotton placed in this tube served as a filter. In each solution, the first portion of fluid drawn through the cotton was discarded. Samples of appropriate size were placed in each of two weighing bottles and in a pycnometer. One sample was used for the determination of amine content, one for theophylline content, and that in the pycnometer for the determination of specific gravity.

Amine content was determined by titration with 0.1 *N* H_2SO_4 using methyl red indicator solution. Theophylline content was determined by the procedure described in the U. S. P. XII under Theophylline Ethylenediamine. The specific gravity was used to convert sample weights to sample volumes in order to calculate molarities.

DISCUSSION

Plotting amine concentrations, in molarity, against the solubility of theophylline, also expressed as molarity, the curves shown in Figs. 1 to 3 were obtained. In Fig. 1, a comparison of the solubilizing effect of four primary amines indicates that for any given molarity below the point of maximum solubility, the solubilizing effect decreases with increase in length of the carbon chain. It is also evident, however, that the maximum solubility increases with increase in length of the carbon chain. Inasmuch as no simple or constant ratio of theophylline to amine concentration was found to hold at any of the several points of maximum solubility, no conclusions have been drawn concerning compound formation.

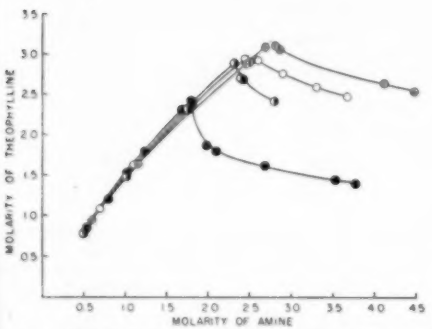

Fig. 1.—Primary amines effect on solubility of theophylline. ●, methylamine; ◑, ethylamine; ○, *n*-propylamine; ⊕, *n*-butylamine.

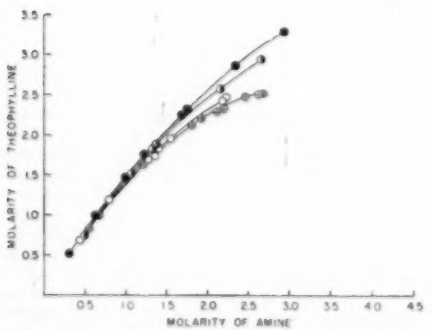

Fig. 2.—Secondary amines effect on solubility of theophylline. ●, dimethylamine; ◑, diethylamine; ○, di-*n*-propylamine; ⊕, di-*n*-butylamine.

In Fig. 2, which depicts the behavior of dialkylamines, it is again evident that at a given amine concentration, the solubilizing effect decreases with increasing length of the carbon chain. With these secondary amines points of maximum solubility were not reached although preliminary observations have indicated that the amine concentrations here employed for di-*n*-propylamine and di-*n*-butylamine are probably approaching the point of maximum solubilization.

In Fig. 3, a comparison of the effect of the methyl amines indicates that for any given concentration of amine below the point of maximum solubility of theophylline, the monomethylamine has the greatest solubilizing action but that the maximum solubility obtained with monomethylamine is not so great as that obtainable with dimethylamine or trimethylamine.

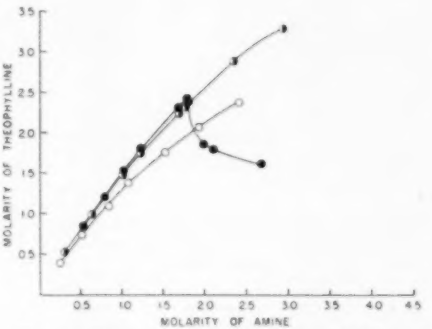

Fig. 3.—The methylamines effect on solubility of theophylline. ●, methylamine; ◑, dimethylamine; ○, trimethylamine.

It may also be seen from Figs. 1 and 2 that in all instances a monoalkylamine has a greater solubilizing effect on theophylline than does the corresponding dialkylamine in the region below that of the maximum solubility of theophylline.

SUMMARY

1. The solubility of theophylline in certain amine-water systems has been determined.

2. By plotting amine molarity against theophylline molarity it has been shown that, as a general rule, in dealing with lower concentrations of amines, the solubility of theophylline increases with increasing amine concentration.

3. In the case of primary amines, the solubility was found to increase with increasing concentration of amine until a point of maximum solubility was reached, after which the solubility decreased with increasing amine concentration. This maximum solubility increased with increasing length of the carbon chain.

4. The molar solubilizing effect of monomethylamine is greater than that of dimethylamine, which, in turn, is more effective than trimethylamine.

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A Bactericidal Spectrum of Some Common Organisms*

By MORRIS OSTROLENK and C. M. BREWER

The reactions of fourteen selected microorganisms to critical germicidal concentrations of twelve chemical compounds are presented. The use of a greater variety of commonly occurring bacteria as an adjunct to conventional germicidal testing methods is briefly discussed.

THE TERM "germicide" is defined as a substance or agent that kills germs. Unless specifically qualified, limits and restrictions on the percentage or types of germs killed do not find a place in this definition; with the exception that it is customary to exclude bacterial spores from the requirements of effective germicidal action. In spite of the fact that interest in germicidal activity extends into the past as far back as our concern with bacteriology itself, and that the difference in susceptibility to germicides was early recognized, with the exception of two microorganisms, criteria of representative resistance of different types of bacteria for germicidal testing purposes have not yet been established. Only from *Micrococcus pyogenes* var. *aureus* and *Salmonella typhosa* have strains been deliberately selected to represent standard resistance of their species to germicidal action. A strain of *M. aureus* was designated by Reddish (3) following the initiatory work of Shippen, and, earlier, by Anderson and McClintic (1) in the case of *S. typhosa*.

The fallacy of placing reliance on the reaction of a single strain of test organism to bactericides as a measure of germicidal potentiality has been recognized since the earliest studies when various bacterial species were used. Among the many who have advocated or utilized different species of microorganisms in more recent studies of germicidal activity, Philbrick (2), Reddish (4), Tilley (5), Walters (6), Zoller and Eaton (7) may be cited. Scientific literature is noticeably lacking in germicidal data on the use of a number of strains of different species of test organisms that have been standardized against one or more chemically defined compounds. For entirely too long, tests limited to the reactions of *S. typhosa* (Hopkins) and *M. aureus* toward antibacterial preparations under rigid sets of conditions have been widely exploited and accepted as a means of estimating antiseptic and disinfectant efficacy. Standardization of tests offering comparison under arbitrary conditions has generally been favored over measurement by practical accomplishment. Neither aspect can properly be neglected. An ideal standardized method that can compare antibacterial potentialities and at the same time serve as an indication of efficacy has long been sought. Such a test is yet to be realized. The use of a Gram-negative organism (*S. typhosa*) relatively susceptible to germicidal substances, together with a comparatively resistant Gram-

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positive strain of microorganism (*M. aureus*), may afford a reasonable amount of justification for estimating the limits of effective concentrations of germicides to be used as disinfectants on inanimate surfaces. The established precedent of recommending a disinfectant for practical application at concentrations equivalent to twenty times its phenol coefficient value, which is theoretically comparable to the use of 5 per cent phenol, provides, in general, an acceptable margin of safety. When, however, germicides are used as antiseptics on living mammalian tissue where toxicity of the agent may be of greater importance than germicidal potency, no such factor of bactericidal safety can feasibly be employed.

In order to obtain more explicit information on germicidal testing, strains from a number of different microorganisms were selected and tested against several well-recognized chemical compounds. The significance of the work lies in the uniform selection and testing of various organisms against a variety of antibacterial chemical agents. In this respect only is the presented material unique. No original concept motivated this accumulation of data. No attempt to introduce innovations in currently used methods or recommendation of a particular procedure is intended. These data were obtained solely for our own information and are offered only as of possible interest to others engaged in germicidal testing. The limited results omit many important groups of both organisms and chemical compounds, and thus belie the title. However, the demonstration of a complete spectrum of germicidal values is obviously beyond the scope of a single piece of work.

EXPERIMENTAL

Methods and Materials.—Organisms were taken from nutrient agar slants and carried continuously in an unadjusted broth containing only 2% trypticase. Its *p*H was 6.6–6.8 after sterilization. All organisms were transferred daily, using one 4-mm. loopful of inoculum, and grown at 37°. For subculture after exposure to germicidal agents, 15-ml. amounts of a fluid thioglycollate medium were used. Selection of 14 representative test organisms from nine genera was based on reaction to phenol at 20°. Test organisms were selected as representing the more resistant strains of their respective genera and on the basis of uniformity of reaction on repeated tests against phenol. Each test organism, except *S. typhosa* (Hopkins), *M. aureus* (209), and *Serratia marcescens*, was selected from groups composed of from four to 15 strains. The selected test organisms were grown and transferred as before. The number of viable cells per ml. was estimated for each test culture. These counts were made by seeding serial dilutions in nutrient agar. Counts were made on four separate occasions and each was made in triplicate. The estimated number of viable cells for each organism remained quite uniform throughout the tests. The names, designations and viable counts per ml. are given in Table I. The chemical compounds used in the tests are given in Table I-A.

TABLE I.—TEST ORGANISMS

Organism	Designation	Bacterial Count per Ml.[a]
Micrococcus pyogenes var. *aureus*	*S.a*	725,000,000
Micrococcus pyogenes var. *albus*	*S-2*	650,000,000
Micrococcus pyogenes sp.	*S-1*	400,000,000
Micrococcus pyogenes (human throat)	*E-2*	290,000,000
Streptococcus (viridans type)	*S-3*	41,500,000
Streptococcus fecalis	*St. fec.*	560,000,000
Corynebacterium hofmanni	*C. hof.*	147,500,000
Corynebacterium ovis	*C. ovi.*	300,000,000
Eberthella typhosa	*E. ty.*	990,000,000
Pseudomonas aeruginosa	*Ps. aer.*	3,600,000,000
Escherichia coli	*E. col.*	1,500,000,000
Aerobacter aerogenes	*A. aer.*	1,300,000,000
Serratia marcescens	*S. mar.*	1,500,000,000
Proteus vulgaris	*P. vul.*	750,000,000

[a] Trypticase broth (24 hours at 37°).

TABLE I-A.—COMPOUNDS USED FOR TESTING RELATIVE RESISTANCE AND SUSCEPTIBILITY OF TEST ORGANISMS[a]

Compound	Abbreviation
Alcohol (ethyl)	Et alc
Alcohol (Isopropyl)	I P alc
Hydrochloric acid	HCl
Hydrogen peroxide	H_2O_2
Iodine (aqueous sol.)	I_2
Saponated Cresol solution	Cresol
Mercuric chloride	HgCl
Carbolic acid	Phenol
Phenyl mercuric acetate	PMA
Phenyl mercuric nitrate	PMN
Silver nitrate	$AgNO_3$
Sodium hydroxide	NaOH

[a] Dilutions in the tables represent absolute concentrations except under the caption "Cresol," where concentrations of the U. S. P. solution are represented.

The testing technique employed was the phenol coefficient type of method (drop method). The procedure differed from that described in the A. O. A. C. Methods of Analysis[1] only in the media used, and the four-day period of subculture incubation.

Reactions of the organisms to the various chemicals were tested at 20° and 37°. The results are arranged in tables, one for each of the 12 chemical reagents, and show survival or susceptibility of each organism after five, ten, and fifteen minutes' exposure over the range of critical dilutions of the germicides. The recorded results shown as single tests actually represent compilations of all tests for any one organism against any one compound, with the occasional exception of an obviously aberrant result. The individual trial tests varied from 4 to 27, as indicated by the figure at the foot of each column

[1] The same procedure originally described in U. S. Department of Agriculture Circular No. 198.

With the exception of phenol, which was utilized frequently to check the resistance of the test organisms, relatively large numbers of trial tests were performed when inconsistent results were obtained. Growth in the subculture, indicating survival of the exposed organism is designated "+" when this result was obtained in no fewer than two-thirds of the trial tests. Similarly, when no growth occurred in the subcultures in at least two-thirds of the tests the result is designated "0." When inconsistencies resulted at any one concentration time period so that positive and negative results occurred about equally, actually between one-third and two-thirds of the trial tests, the result is designated as "0/+."

In each table, the reactions of the individual test organisms have been arranged in a descending order of resistance when tested at 20°. The summarized results are given in Tables II–XIII.

RESULTS

From the compiled data offered in the tables all the information accumulated in this study can be obtained. Several observations, however, appear to warrant special attention. Most striking is the interchange of relative susceptibility of the organisms to different reagents. Except in the case of the mercurial compounds, where sufficiently concentrated solutions to effect germicidal action could not be obtained for many of the organisms, the relationship of resistance was not the same toward any two chemical reagents. Considering the Gram-positive and Gram-negative groups independently, not only was every organism found at one or the other extreme on the scale of comparative resistance in its reactions to different compounds, but in several instances the organism most susceptible to one compound was the most resistant to another. As examples: The staphylococcus designated "*S-2*" resisted a higher concentration of cresol than any other organism but was the most susceptible to the action of iodine. In the Gram-negative group *P. vulgaris* exhibits the most resistance to hydrochloric acid and *Ps. aeruginosa* the least; whereas, tested against iodine the reverse situation is encountered.

From the tables can be obtained the range of germicidal dilutions, selective or nonselective action, the comparative reactions of the Gram-positive group and Gram-negative group, and the differences in temperature reactions, elicited by the different compounds.

It can be observed from the charts that some germicidal compounds have a marked selective action against the Gram-negative group of organisms. This is the case with sodium hydroxide and, within a very narrow range, the two alcohols. The converse was found when silver nitrate was used as a testing

TABLE II.—PHENOL

		Test Organisms — Gram-positive											
	Diln.	*St. fec.*	*S-2*	*S-a*	*S-1*	*C. kaf.*	*S-3*	*C. ovi.*	*E-2*
20°	1:50	0 0 0	...	0 0 0	...	0 0 0	...	...	...
	1:60	++0/+	+0 0	+0 0	0/+0 0	0/+0 0	0/+0 0	0 0 0	0 0 0 0
	1:70	+++	+++	++0/+	+ ++	+ +0/+	+ 0/+0	+0 0	0 +0 0
	1:80	...	...	+++	...	+ ++	+ + 0/+	++ 0	+ ++0/+
	1:90	...	...	...	...	...	+ + +	...	+++
No. of tests		14	6	27	6	6	9	6	9
37°	1:70	0 0 0	0 0 0	...	...	...	...	...	...
	1:80	+0/+0	+0 0	0 0 0	0 0 0	...	...	...	...
	1:90	++ +	++0/+	+0/+0	+0 0	0 0 0	...	0/+0 0	0 0 0 0
	1:100	...	+++	++ +	+++	+0 0	0/+0 0	+0/+ 0	+0 0
	1:110	...	...	...	...	++0/+	+ +0	+ + +	++0/+
	1:120	...	...	...	...	+++	+ ++	...	+++
No. of tests		9	6	19	6	5	10	5	11
		Gram-negative							
	Diln.	*E. col.*	*S. mar.*	*A. aer.*	*Ps. aer.*	*E. typhosa*	*P. vul.*
		...	...	...	...	...	...
20°	1:60	0/+0 0	0 0 0	0 0 0	0 0 0	...	...
	1:70	+ ++	+0 0	0/+0 0	0/+0 0	0 0 (	0 0 0
	1:80	...	+++	+ + 0	+ +0/+	+0/+ 0	+0 0
	1:90	...	...	+ ++	+ ++	++ +	+++
No. of tests		16	5	5	11	18	5
37°	1:80	0 0 0	...	...	...	...	...
	1:90	+0/+0	...	...	...	...	...
	1:100	++ 0/+	0 0 0	...	...	...	...
	1:110	++ +	++0	0 0 0	0/+0 0	...	...
	1:120	...	++0/+	0/+0 0	+ +0	...	0 0 0
	1:130	...	+++	+ 0 0	+ +0/+	0 0 0	0/+0 0
	1:140	...	...	+ +0/+	+ ++	+0 0	+ 0 0
	1:150	...	...	+ ++	...	++ 0	+ +0
	1:160	...	...	...	...	++ 0/+	+ ++
	1:170	...	...	...	...	++ +	...
No. of tests		10	6	5	9	11	5

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE III.—PHENYLMERCURIC NITRATE

		Test Organisms — Gram-positive							
	Diln.	*S-a*	*St. fec.*	*S-3*	*S-1*	*S-2*	*E-2*	*C. hof.*	*C. ovi.*
20°	1:1350	+++	+++	+++	+++	+++	+++	+++	++0/+
	1:2700	...	...	...	...	...	...	...	+++
No. of tests		8	8	7	6	6	7	6	8
37°	1:1350	+++	+++	+++	+++	+++	0 0 0	...	...
	1:2700	...	...	...	...	...	+++	0 0 0	. 0 0 0
	1:6750	...	...	...	...	...	...	+++	+++
No. of tests		8	8	7	6	6	7	6	8

		Gram-negative					
	Diln.	*E. col.*	*P. vul.*	*S. mar.*	*Ps. aer.*	*E. typhosa*	*A. aer.*
20°	1:1350	+++	+++	+++	+++	+++	+++
No. of tests		4	5	5	5	5	4
37°	1:1350	++0/+	+0/+0	+0/+0	+0 0	+0 0	0/+0 0
	1:2700	+++	++ +	++ 0/+	++0	+0/+0	+ 0 0
	1:6750	...	...	++ +	+++	+0/+0/+	+ +0/+
	1:13,500	...	...	...	...	++ +	+ ++
No. of tests		4	5	5	5	5	4

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE IV.—PHENYLMERCURIC ACETATE

		Test Organisms — Gram-positive							
	Diln.	*S-1*	*St. fec.*	*S-3*	*S-a*	*E-2*	*S-2*	*C. hof.*	*C. ovi.*
20°	1:350	+++	++ +	++ +	++ 0/+	0/+ 0 0	0 0	0 0	...
	1:700	...	...	...	++ +	+ 0/+0	+0/+ 0	0/+0 0	0 0 0
	1:1750	...	...	...	...	+ + +	++ + +	0/+0	+0/+0
	1:3500	...	...	...	...	...	...	+ + +	+0/+0/+
	1:5250	...	...	...	...	...	...	...	++ +
No. of tests		7	6	6	6	8	5	5	6
37°	1:350	+++	+0/+0/+	0 0 0	0 0 0	...	...	...	...
	1:700	...	++ +	0/+0 0	0/+ 0/+ 0	0 0 0	0 0 0	...	...
	1:1750	...	...	+ 0/+0	+ + 0/+	0/+ 0 0	+0 0 0	0 0 0	...
	1:3500	...	...	+ + 0/+	+ + +	+ 0 0	++ +	+ 0/+0	0 0 0
	1:5250	...	...	+ + +	...	+ 0 0	...	+ + 0/+	+++ +
	1:7000	...	...	...	...	+ 0 0	...	+ + +	...
	1:8750	...	...	...	...	+ + 0	...	...	...
	1:10,500	...	...	...	...	+ + +	...	...	...
No. of tests		4	5	4	5	5	4	5	4

		Gram-negative					
	Diln.	*E. col.*	*A. aer.*	*Ps. aer.*	*P. vul.*	*E. typhosa*	*S. mar.*
20°	1:350	0/+ 0 0	0 0 0	0 0 0	0 0 0	...	...
	1:700	+ 0 0	+0/+0	0/+0 0	+0 0	0 0 0	0 0 0
	1:1750	+ ++	++ +	+ +0/+	++0/+	++0/+	+0 0
	1:3500	...	...	+ ++	+++	+++	+++
No. of tests		5	5	4	6	4	4
37°	1:1750	0 0 0	...	0 0 0	0 0 0	...	...
	1:3500	+ 0/+0	0 0 0	+0/+0/+	+0 0	...	0 0 0
	1:5250	+ + 0	...	...	...	...	...
	1:7000	+ + +	+0 0	++ 0/+	++0	0 0 0	+0 0
	1:10,500	...	+0 0	++ +	+++	0/+0 0	++0
	1:14,000	...	+0/+0	...	...	+ 0 0	+++
	1:17,500	...	+0/+0	...	...	+ 0 0	...
	1:21,000	...	++ 0/+	...	...	+ 0/+0	...
	1:24,500	...	++ +	...	...	...	...
	1:28,000	...	...	...	...	+ + 0	...
	1:35,000	...	...	...	...	+ + +	...
No. of tests		7	6	7	5	6	5

0 = No growth in subculture.
+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE V.—MERCURIC CHLORIDE

TEST ORGANISMS

Gram-positive

	Diln.	*S-a*	*St. fec.*	*S-1*	*E-2*	*S-3*	*S-2*	*C. kof.*	*C. ovi.*
20°	1:20	+++ + + +	+++	++ 0/+	+0/+ 0/+	0/+0/+0	...	...	
	1:30	... ...	...	++ +	++ +	+ 0/+0/+	...	...	
	1:40	... ...	...	...	...	+ 0/+0/+	0 0 0	0 0 0	
	1:50	... ...	...	...	...	+ + +	0/+0/+0/+	0/+0 0	
	1:60	... ...	...	...	...	...	+ + +	+ 0/+0/+	
	1:70	... ...	...	...	...	...	...	+ + 0/+	
	1:80	... ...	...	...	...	...	...	+ + +	
No. of tests		5	9	4	5	5	4	4	4
37°	1:20	0/+0 0 0/+0 0	++0/+	...	...	...	...	...	
	1:30	0/+0 0 + 0/+0/+	+++	0 0 0	...	...	...	...	
	1:40	+ +0 + + +	...	+0 0	0 0 0	...	...	...	
	1:50	+ +0	...	+0 0	0/+0 0	...	...	...	
	1:60	+ +0/+	...	+0 0	0/+0 0	...	...	...	
	1:70	+ ++	...	+0/+0	+ 0 0	0 0 0	...	00 0	
	1:80	...	...	+0/+0	+ 0/+0	0/+0 0	0 0 0	0/+0 0	
	1:90	...	...	+0/+0	+ + 0	0/+0 0	0/+0 0	+ 0 0	
	1:100	...	...	+0/+0	+ + 0	0/+0 0	0/+0 0	+ 0 0	
	1:200	...	...	+0/+0	+ + 0/+	+ 0/+0	+ + +	+ + 0	
	1:300	...	...	++ 0	+ + +	+ 0/+0	...	+ + 0/+	
	1:400	...	...	++ 0/+	...	+ 0/+0	...	+ + +	
	1:500	...	...	++ +	...	+ + 0	...	...	
	1:600	...	...	...	...	+ + +	...	...	
No. of tests		7	7	7	7	6	6	6	6

Gram-negative

	Diln.	*E. col.*	*S. mar.*	*A. aer.*	*E. typhosa*	*P. vul.*	*Ps. aer.*
20°	1:20	++ 0/+	+ +0/+	+ + 0/+	0/+0/+0	0/+0 0	...
	1:30	++ +	+ +0/+	+ + 0/+	+ + 0/+	+ 0/+0	...
	1:40	...	+ ++	+ ++	+ + +	+ + 0	...
	1:50	...	...	...	...	+ + 0/+	...
	1:60	...	...	...	...	+ + +	...
	1:80	...	...	...	...	...	0 0 0
	1:90	...	...	...	...	...	0/+0 0
	1:100	...	...	...	...	...	0/+0 0
	1:200	...	...	...	...	...	+ 0/+0/+
	1:300	...	...	...	...	...	+ + 0/+
	1:400	...	...	...	...	...	+ + +
No. of tests		5	5	5	5	4	5
37°	1:20	0 0 0	0/+0 0	...	...	...	...
	1:30	0/+0 0	...	...	...	...	...
	1:40	+ 0 0	0/+0 0	...	...	...	...
	1:50	+ +0	...	0 0 0	0 0 0	...	...
	1:60	+ +0/+	+ 0 0	0/+0 0	0/+0 0	...	...
	1:70	+ ++	+ 0 0	0/+0 0	0/+0 0	0 0 0	...
	1:80	...	+ 0 0	0/+0/+0	0/+0 0	0/+0 0	
	1:90	...	+ 0 0	+ 0/+0	+ 0 0	0/+0 0	...
	1:100	...	+ +0	+ 0/+0	+ 0 0	+ 0 0	...
	1:200	...	+ ++	+ + +	+ + 0	+ 0 0	...
	1:300	...	...	...	+ + 0	+ + 0	0 0 0
	1:400	...	...	...	+ + 0/+	+ + +	0/+0 0
	1:500	...	...	...	+ + +	...	0/+0 0
	1:600	...	...	...	...	...	0/+0 0
	1:700	...	...	...	...	...	0/+0/+0
	1:800	...	...	...	...	...	+ 0/+0
	1:900	...	...	...	...	...	+ + +
No. of tests		5	5	7	5	7	6

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE VI.—ALCOHOL

| | | Test Organisms — Gram-positive | | |

TABLE VII.—ISOPROPYL ALCOHOL—(*Cont'd from p. 100*)

		Test Organisms — Gram-negative					
	Per Cent	*S. mar.*	*E. col.*	*Ps. aer.*	*A. aer.*	*P. vul.*	*E. typhosa*
20°	30	0 0 0	...	...	...	...	...
	28	+0 0	0 0 0	0 0 0	0 0 0	0 0 0	...
	26	+++	++ +	+0/+0	+0 0	0/+0 0	0 0 0
	24	...	...	++ +	+++	+ + +	+++
No. of tests		5	5	5	4	5	5
37°	18	0 0 0	0 0 0	...	...	...	...
	16	+0 0	+0/+0	0 0 0	0 0 0	0 0 0	0 0 0
	14	+++	++ +	++ 0	+0 0	+ 0/+0	+0 0
	12	...	...	++ +	+++	+ + +	+++
No. of tests		5	5	5	4	5	5

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE VIII.—CRESOL (U. S. P.)

		Test Organisms — Gram-positive							
	Dilu.	*S-2*	*S-1*	*E-2*	*S-a*	*St. fec.*	*C. ovi.*	*C. hof.*	*S-3*
20°	1:50	0 0 0	...	...	...	...	...	...	...
	1:60	++0/+	...	...	...	...	...	...	...
	1:70	+++	...	...	...	...	...	...	...
	1:80	...	0 0 0	0 0 0	...	...	...	...	...
	1:90	...	+0 0	+0 0	...	...	...	...	...
	1:100	...	++ 0	+0/+0	0 0 0 0 0 0 0 0 0 0				...
	1:150	...	++ +	++ +	+0/+0 + 0 0 0/+0 0 0/+0 0				...
	1:200	...	...	...	++ + + + + + + + + +				...
	1:500	...	...	...	...	...	...	...	0/+0 0
	1:600	...	...	...	...	...	...	...	+ 0/+0
	1:700	...	...	...	...	...	...	...	+ + 0
	1:800	...	...	...	...	...	...	...	+ + +
No. of tests		6	7	6	6	5	5	6	7
37°	1:90	+0 0	...	...	...	...	...	...	...
	1:100	++0	...	...	...	...	...	...	...
	1:150	+++	...	0 0 0	...	0 0 0	...	...	...
	1:200	...	+0/+0	+0 0	...	0/+0 0	...	...	...
	1:250	...	++ 0/+	++ 0/+	0 0 0	+ 0/+0	0 0 0	0 0 0	...
	1:300	...	++ +	++ +	+0/+0	+ + +	+ 0/+0	+ 0/+0	...
	1:350	...	...	...	++ 0	...	+ + +	+ + 0/+	...
	1:400	...	...	...	++ +	...	...	+ + +	...
	1:800	...	...	...	...	...	...	...	0 0 0
	1:1000	...	...	...	...	...	...	...	0/+0 0
	1:1200	...	...	...	...	...	...	...	+ 0/+0
	1:1400	...	...	...	...	...	...	...	+ + 0
	1:1600	...	...	...	...	...	...	...	+ + +
No. of tests		7	7	7	6	6	5	6	8

		Gram-negative					
	Dilu.	*E. col.*	*A. aer.*	*E. typhosa*	*S. mar.*	*Ps. aer.*	*P. vul.*
20°	1:80	0 0 0	0 0 0	...	...	...	...
	1:90	+0 0	0/+0 0	...	...	...	...
	1:100	+0 0	+ 0 0	0 0 0	0 0 0	0 0 0	...
	1:150	++ +	+ + 0	+0 0	+0 0	+0 0	0 0 0
	1:200	...	+ + +	+++	+++	+++	++ +
No. of tests		4	4	4	4	6	5
37°	1:150	0 0 0	...	...	...	0 0 0	0 0 0
	1:200	+0/+0	0 0 0	...	0 0 0	+0 0	0/+0 0
	1:250	++ +	+ 0/+0	0 0 0	+0 0	+++	+ +0/+
	1:300	...	+ + +	++0/+	+++	...	+ ++
	1:350	...	...	+++	...	...	...
No. of tests		5	5	5	5	6	7

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE IX.—HYDROCHLORIC ACID

	Dilu. of 36.3% Soln.	Conc. Gm./Ml. HCl	St. fec.	S-a	S-1	S-2	C. ovi.	S-3	E-2	C. hof.
			Test Organisms—Gram-positive							
20°	1:40	1:93	0 0 0	...	...	...	...	...	...	...
	1:50	1:116	+0 0	0 0 0	...	...	...	...	...	...
	1:60	1:140	+0 0	+0 0	...	...	...	...	...	...
	1:70	1:163	+0/+0	+0 0	0 0 0	...	...	...	...	...
	1:80	1:186	++ 0	+0/+0/+	0/+0	...	...	...	...	...
	1:90	1:210	++ 0	++ +	0/+0 0	...	...	...	...	...
	1:100	1:233	++ 0	...	0/+0 0	0 0 0 0 0 0	...	...	...	...
	1:150	1:350	++ +	...	+ 0 0	+0 0 0/+0 0	0 0 0	0 0 0	0 0 0	...
	1:200	1:467	...	...	+ ++	+0/+0 + 0/+0/+	0/+0 0	0/+0 0	0 0 0	
	1:250	1:584	...	...	...	++ + + + 0/+	+ 0/+0	+ 0 0	+ 0/+0/+	
	1:300	1:700	...	...	...	... + + +	+ 0/+0	+ 0/+0	+ ++	
	1:350	1:817	...	...	...	... ...	+ + 0/+	+ 0/+0	...	
	1:400	1:934	...	...	...	... ...	+ + +	+ + 0/+	...	
	1:500	1:1168	...	...	...	... ...	... ...	+ + +	...	
No. of tests			5	8	8	7	8	10	12	10
37°	1:100	1:233	...	0 0 0	0 0 0	...	...	...	...	...
	1:150	1:350	...	+0 0	...	...	...	...	...	...
	1:200	1:467	...	++0/+	0/+0 0	...	...	...	...	...
	1:250	1:584	0 0 0	+++	...	...	...	...	...	...
	1:300	1:700	0/+0 0	...	+ 0 0	...	...	...	...	...
	1:350	1:817	+ 0 0	...	...	...	...	...	...	...
	1:400	1:934	+ 0/+0/+	...	+ 0 0	0 0 0 0 0 0	...	0 0 0	0 0 0	
	1:500	1:1168	+ + +	...	+ 0/+0	+0 0 0/+0 0	...	0/+0 0	0/+0 0	
	1:600	1:1401	...	...	+ + 0	+0/+0 0/+0 0	...	0/+0 0	0/+0/+0	
	1:700	1:1635	...	...	+ + +	++ + + 0 0	0 0 0	+ 0 0	+ 0/+0	
	1:800	1:1869	...	...	...	... + + 0/+	+ 0 0	+ 0 0	+ + +	
	1:900	1:2102	...	...	...	... + + +	+ 0/+0	+ + 0	...	
	1:1000	1:2336	...	...	...	... ...	+ 0/+0	+ + 0	...	
	1:1100	1:2570	...	...	...	... ...	...	+ + 0/+	...	
	1:1200	1:2803	...	...	...	... ...	+ + 0	+ + +	...	
	1:1300	1:3037	...	...	...	... ...	+ + +	...	...	
No. of tests			8	6	7	7	11	11	9	8

	Dilu. of 36.3% Soln.	Conc. Gm./Ml. HCl	P. vul.	E. col.	A. aer.	S. mar.	E. typhosa	Ps. aer.
			Gram-negative					
20°	1:80	1:186	0 0 0	...	...	...	...	...
	1:90	1:210	0/+0 0	0 0 0	...	...	...	...
	1:100	1:233	0/+0 0	0/+0 0	...	...	...	...
	1:150	1:350	0/+0/+0	+ 0/+0	0 0 0	0 0 0	0 0 0	...
	1:200	1:467	+ 0/+0	+ + +	0/+0/+0	0/+0 0	0/+0 0	...
	1:250	1:584	+ + 0/+	...	+ 0/+0	...	...	...
	1:300	1:700	+ + +	...	+ + 0	+ 0/+0	+ 0 0	...
	1:350	1:817	...	...	+ + +	...	...	...
	1:400	1:934	...	...	...	+ + 0/+	+ 0/+0/+	0 0 0
	1:500	1:1168	...	...	...	+ + +	+ + +	0/+0 0
	1:600	1:1401	...	...	...	...	...	0/+0 0
	1:700	1:1635	...	...	...	...	...	0/+0/+0
	1:800	1:1869	...	...	...	...	...	+ 0/+0/+
	1:900	1:2102	...	...	...	...	...	+ + 0/+
	1:1000	1:2336	...	...	...	...	...	+ + +
No. of tests			11	7	11	8	14	13
37°	1:90	1:210	...	0 0 0	...	...	...	...
	1:100	1:233	...	0/+0 0	...	...	...	...
	1:150	1:350	...	0/+0 0	...	...	...	...
	1:200	1:467	...	+ 0/+0/+	...	...	...	...
	1:250	1:584	...	+ + +	...	...	...	...
	1:300	1:700	0 0 0	...	0 0 0	...	...	...
	1:400	1:934	0/+0 0	...	0/+0/+0	0 0 0	...	...
	1:500	1:1168	+ 0/+0	...	0/+0/+0	0/+0 0	...	...
	1:600	1:1401	+ 0/+0	...	0/+0/+0	0/+0/+0	0 0 0	...
	1:700	1:1635	+ + 0	...	+ 0/+0/+	+ 0/+0	+ 0 0	...
	1:800	1:1869	+ + +	...	+ + +	+ 0/+0/+	+ 0/+0	...
	1:900	1:2102	...	...	...	+ + +	+ + 0/+	...
	1:1000	1:2336	...	...	...	...	+ + +	0 0 0
	1:1100	1:2570	...	...	...	...	...	0/+0/+0
	1:1200	1:2803	...	...	...	...	...	0/+0/+0
	1:1300	1:3037	...	...	...	...	...	+ + +
No. of tests			11	7	11	12	14	10

TABLE X.—SODIUM HYDROXIDE

(18.9 *N*—Sat. Sol.)

	Diln. of Sat. Soln.	Conc. Gm./Ml. NaOH	Test Organisms — Gram-positive: *S-2*	*C. hof.*	*S-a*	*C. ovi.*	*E-2*	*St. fec.*	*S-1*	*S-3*
20°	1:10	1:13.2	0 0 0	...	...	...	...	...	...	...
	1:20	1:26.5	0/+0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	...
	1:30	1:39.7	+ 0/+0	+0/+ 0/+	+0/+0/+	0/+0 0	0/+0 0	0/+0 0	0/+0 0	...
	1:40	1:52.9	+ + +	++ +	++ +	+ ++	+ + 0/++	0/+0	+ 0 0	...
	1:50	1:66.2	...	...	...	...	...	+ 0/+0	+ +0/+	...
	1:60	1:79.4	...	...	...	...	+ + 0/++	0/+0/+	+ ++	...
	1:70	1:92.6	...	...	...	...	+ + + +	+ +	...	...
	1:90	1:119.1	...	...	...	...	...	...	...	0 0 0
	1:100	1:132.2	...	...	...	...	...	...	...	0/+0 0
	1:200	1:264.4	...	...	...	...	...	...	...	+ ++
No. of tests			6	6	9	5	6	6	6	6
37°	1:30	1:39.7	...	0 0 0	...	...	...	...	...	...
	1:40	1:52.9	0 0 0	+0 0	...	...	0 0 0 0 0 0	...	...	...
	1:50	1:66.2	0/+0 0	+0 0	...	0 0 0	0/+0 0 0/+0 0	0 0 0	0 0 0	...
	1:60	1:79.4	+ 0/+0	+0/+0/+	0 0 0	+0/+ 0/+	0/+0 0 0/+0 0	0/+0 0	0/+0 0	...
	1:70	1:92.6	+ 0/+0/+	++ 0/+	++ 0	+0/+ 0/+	+ 0 0 +	0/+0/+	0/+0 0	...
	1:80	1:105.8	+ 0/+0/+	...	++ 0	+0/+ 0/+	+ + + +	+ +	+ 0 0	...
	1:90	1:119.1	+ + +	++ 0/+	++ +	++ 0/+	...	...	+ +0	...
	1:100	1:132.2	...	++ +	...	++ +	...	...	+ +0/+ 0	0 0
	1:200	1:264.4	...	...	...	...	...	...	+ ++ +	+0
	1:300	1:396.6	...	...	...	...	...	...	...	+ ++
No. of tests			6	6	8	5	7	5	5	6

	Diln. of Sat. Soln.	Conc. of NaOH	Gram-negative: *P. vul.*	*A. aer.*	*S. mar.*	*Ps. aer.*	*E. typhosa*	*E. col.*
20°	1:200	1:264.4	+ 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	1:300	1:396.6	+ +0	+ +0/+	+0/+0/+	+0/+0	+0/+ 0	0/+0/+0
	1:400	1:528.8	+ +0/+	+ ++	++ 0/+	++ +	++ +	+ + +
	1:500	1:662.0	+ ++	...	++ +	...	...	...
No. of tests			7	6	6	6	7	7
37°	1:100	1:132.2	...	0 0 0	...	...	...	...
	1:200	1:264.4	0 0 0	0/+0 0	...	0 0 0	...	0 0 0
	1:300	1:396.6	0/+0/+0	+ 0 0	0 0 0	0/+0 0	0 0 0	0/+0 0
	1:400	1:528.8	+ + 0	+ +0/+	+0 0	+ +0	0/+0 0	+ + 0/+
	1:500	1:662.0	+ + +	+ ++	++ 0/+	+ ++	+ + 0/+	+ + +
	1:600	1:793.2	...	...	++ +	...	+ + 0/+	...
	1:700	1:925.4	...	...	...	...	+ + +	...
No. of tests			6	7	6	6	8	7

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XI.—IODINE

	Diln.	Test Organisms — Gram-positive: *C. hof.*	*S-1*	*St. fec.*	*C. ovi.*	*S-a*	*E-2*	*S-3*	*S-2*
20°	1:5000	0 0 0 0 0 0	...	...	...	...	...	...	...
	1:6000	0/+0 0	0/+0 0	0 0 0	...	...	...	...	...
	1:7000	+ +0/+	0/+0 0	+0 0	0/+0 0	0 0 0	...	...	...
	1:8000	+ + +	+ + +	+++	+ + + ++	+	0 0 0	...	...
	1:9000	...	...	...	...	...	0/+0/+0/+	...	...
	1:10,000	...	...	...	...	...	+ + +	0 0 0	0 0 0
	1:11,000	...	...	...	...	...	...	0/+0/+0/+	0/+0/+0
	1:12,000	...	...	...	...	...	...	+ + +	0/+0/+0
	1:13,000	...	...	...	...	...	...	...	+ + 0/+
	1:14,000	...	...	...	...	...	...	...	+ + 0/+
	1:15,000	...	...	...	...	...	...	...	+ + +
No. of tests		7	6	6	5	7	5	6	6

(*Cont' on p. 104*)

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XI.—IODINE—(*Cont'd from p. 103*)

| | | Test Organisms — Gram-positive | | |

TABLE XII.—HYDROGEN PEROXIDE—(*Cont'd from p. 104*)

		Test Organisms — Gram-negative					
	Per Cent	*S. mar.*	*A. aer.*	*Ps. aer.*	*P. vul.*	*E. col.*	*E. typhosa*
20°	10	0 0 0	...	...	...	...	...
	9	+0/+0	...	...	...	...	...
	8	++ +	...	...	...	...	...
	4	...	0 0 0	...	...	...	...
	3	...	+0 0	0 0 0	0 0 0	...	...
	2.5	...	...	+0 0	0/+0 0	0 0 0	...
	2	...	+0 0	+0/+ 0	+ 0 0	+0 0	0 0 0
	1.5	...	...	++ +	+ +0/+	+0 0	0/+0 0
	1	...	+++	...	+ ++	+0/+0	+ +0/+
	0.5	...	...	...	...	++ +	+ ++
No. of tests		8	8	6	7	7	7
37°	7	0 0 0	...	...	...	...	...
	6	+0 0	...	...	...	...	...
	5	+0 0	...	...	...	...	...
	4	+0/+0	...	...	...	...	
	3	++ 0	...	...	...	...	...
	2	++ 0/+	...	0 0 0	...	...	...
	1.5	++ +	0 0 0	0/+0 0	0 0 0	...	...
	1	...	0/+0 0	+ 0 0	0/+0 0	0 0 0	0 0 0
	0.5	...	+ 0/+0	+ ++	+ +0/+	0/+0 0	0/+0 0
	0.4	...	+ + 0/+	...	+ ++	+ 0 0	+ 0 0
	0.3	...	+ + +	...	...	+ 0/+0	+ 0/+ 0
	0.2	...	...	...	...	+ + 0/+	+ + 0
	0.1	...	...	...	...	+ + +	+ + +
No. of tests		7	6	7	6	7	7

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

TABLE XIII.—SILVER NITRATE

		Test Organisms — Gram-positive								
	Diln.	*C. ovi.*	*St. fec.*	*C. hof.*	*S-1*	*E-2*	*S-a*	*S-2*	*S-3*	
20°	1:500	0 0 0	0 0 0	0 0 0	...	...	...	...	...	
	1:1000	0/+0 0	0/+0 0	0/+0 0	0 0 0	0 0 0	...	...	...	
	1:2000	0/+0 0	0/+0 0	0/+0 0	+0/+ 0	0/+0 0	0 0 0	...	...	
	1:4000	+ 0 0	+ 0 0	+ 0 0	++ 0/+	0/+0 0	0/+0	0 0 0	...	
	1:6000	+ 0/+0/+	+ +0	+ 0/+0	++ +	+ 0 0	+ 0	0 0 0/+0	...	
	1:8000	+ + +	+ ++	+ + 0/+	...	+ 0 0	+ 0	0 + 0	...	
	1:10,000	...	...	+ + +	...	+ +0/+	+	+ 0 + 0/+0/+	0 0 0	
	1:12,000	...	...	...	...	+ ++	+	++ + + +	0/+0/+0	
	1:14,000	...	...	...	...	...	...	...	+ 0/+0/+	
	1:16,000	...	...	...	...	...	...	...	+ + 0/+	
	1:18,000	...	...	...	...	...	...	...	+ + +	
No. of tests		5	5	5	6	6	5	6	6	

		Gram-negative					
	Diln.	*E. col.*	*Ps. aer.*	*P. vul.*	*A. aer.*	*S. mar.*	*E. typhosa*
20°	1:500	0 0 0	...	...	...	...	...
	1:1000	0/+0 0	0 0 0	...	...	...	...
	1:2000	0/+0 0	0/+0 0	0 0 0	0 0 0	0 0 0	0 0 0
	1:4000	+ 0/+0	+ 0 0	0/+0/+0	+0 0	+0 0	0/+0 0
	1:6000	+ + +	+ +0/+	+ 0/+0	+0/+0	+0/+0	+ 0 0
	1:8000	...	+ ++	+ + +	++ +	++ 0/+	+ 0 0
	1:10,000	...	...	...	...	++ +	+ 0/+0
	1:12,000	...	...	...	...	...	+ 0/+0
	1:14,000	...	...	...	...	...	+ + +
No. of tests		6	5	5	5	5	5

0 = No growth in subculture.
\+ = Growth in subculture.
0/+ = Growth in subculture in approximately 50% of tests.
The three parallel figures in each column indicate 5-, 10-, and 15-minute test periods, respectively.

agent at 37°. Silver nitrate results at 37° appear in Table XIII, since results on only four organisms were obtained in this instance.

Although susceptible to the chemical reagents at higher dilutions when tested at 37°, as compared with the 20° test, a general conformity of relative resistance is demonstrated. However, in tests against every compound one or more organisms, to a greater or less degree, distorted the relationship of resistance at the two different temperatures. In contrast to the other compounds used as test reagents, critical concentrations of iodine were either equally effective at both test temperatures for the Gram-positive organisms or required stronger concentrations at the higher temperature for the Gram-negative bacteria. Also, it should be noted that germicidal efficiency of a compound cannot be compared with another on a dilution basis. For example, although the mercury compounds were utilized at weak dilutions, the generally nongermicidal results were nonetheless based upon the maximum concentrations obtainable in solution. The two alcohols, on the other hand, while requiring relatively strong concentrations to effect germicidal action, not only killed all the test organisms within a narrow range of dilution, but could be utilized at much greater concentrations should necessity demand. The general utility as germicides of the two latter compounds, therefore, far exceeds that of the three mercury products subjected to test. The germicidal impotency of the mercurial substances prevented their use even for detecting relative resistance among the organisms. The differences in reaction of the organisms to the alcohols fell within such a narrow range of effective dilutions that these substances are not particularly suitable for determining relative resistance. On the contrary, there was marked difference in the reactions of the various organisms to hydrochloric acid. No two organisms succumbed to the same concentration; and the frequency with which the individual strains survived a wide range of dilutions at the different time intervals renders this compound a selective agent worthy of consideration. In contrast, was the reaction of the organisms to sodium hydroxide. Within both the Gram-positive and Gram-negative groups there was, in general, very little difference in resistance. However, the Gram-negative organisms elicited the striking characteristic of succumbing to dilutions of this compound far weaker than the concentrations generally withstood by the Gram-positive group.

DISCUSSION

The germicidal values and the apparent relationship of individual resistance of strains found in this study were obtained only under the circumstances peculiar to the arbitrary conditions employed. The use of any other method of test or change of media would have resulted in different values and different ratios of resistance. Had any compound other than phenol been employed for selecting the test organisms, a different choice of strains would probably have resulted. This is apparent from the changing resistance relationship among the four closely related species of staphylococci on their exposure to different types of reagents. Likewise, exposure of the different organisms to two closely related compounds results in a rearrangement of the order of resistance. The reactions of the Gram-negative bacteria to mercuric chloride and phenyl mercuric acetate and changes in the relative reactions of the organisms to ethyl and isopropyl alcohol illustrate this.

The results emphasize again the inadequacy of the conventional phenol coefficient test to indicate the selectivity or nonselectivity of a germicide. It can be noted that the effective concentration of a compound might vary as much as tenfold, depending upon the organism used in the test. It may appear that use of a phenol coefficient type of test in this work, with certain arbitrary features, detracts from the value of the results. However, as an indication of the unpredictable resistance of various organisms, it was essential that a consistent method of treatment, both in the selection and testing, be used throughout. The procedure chosen seemed to offer the most convenient means of obtaining a considerable amount of data. Its employment does not imply its endorsement as a germicidal test, or that phenol is a desirable selective reagent; in fact, the results indicate the reverse. The most significant deduction to be gained from the accumulated data is the impossibility of adequately determining germicidal activity with one representative test organism by a single standardized type of test. If so much is evident from *in vitro* tests of germicidal potentiality, it becomes acutely manifest that the multiplicity of factors involved in antiseptic efficacy precludes the use of standard methods except for crude preliminary indications of antibacterial possibilities.

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Digitalis. VIII. The Baljet Reaction, Digitoxin and Digitoxigenin*

By FREDERICK K. BELL and JOHN C. KRANTZ, JR.

The Baljet reagent has been used in a quantitative study of the color reaction of digitoxigenin and digitoxin with that reagent. The results reported in this paper indicate the reactivity of the two substances with the Baljet reagent on a molar basis to be essentially the same. If extreme precision is required, however, correction must be made for the effect of the digitoxose portion of the digitoxin molecule.

IN BALJET'S original publication (1) of his color reaction for cardiotonic glycosides he included the observation that the genins also give a positive response to the reaction. This observation gave strong support to his conclusion that the reaction depended upon the structure of the genin portion of the glycoside, especially since he had shown that the sugars themselves gave practically no response under the conditions of the test.

In the course of our studies concerning the quantitative determination of the pure digitalis glycosides (2, 3) by means of our adaptation of the Baljet reaction, we have felt that quantitative data concerning the behavior of the genins would afford useful information concerning the validity of the method and the mechanism of the reactions involved. This seemed especially desirable since the only quantitative data available are those of Hagemeier (4) who, moreover, employed an experimental procedure quite different from that which we have adopted. In this communication we will describe and discuss the results we have obtained in our studies of digitoxigenin.

EXPERIMENTAL

Through the kindness of Dr. E. W. McChesney of the Sterling-Winthrop Research Institute we were fortunate in obtaining a sample of digitoxigenin especially prepared for research studies. This specimen of digitoxigenin is a portion of a product the properties and preparation of which have been described elsewhere (5). For purposes of comparison we have used the U. S. P. Reference Standard Digitoxin.

The experimental method employed was essentially that previously described (6) using the Fisher Electrophotometer with the 525-millimicron green filter and the 3-cc. micro-absorption cells. In the development of the color reaction we have used two procedures: (*a*) our original method in which sodium hydroxide serves as the alkali and (*b*) our recently revised method (7) in which tetraethylammonium hydroxide is the alkali.

A stock solution of digitoxigenin in absolute methanol was prepared to contain 25 mg. per 100 cc. This solution was diluted quantitatively with absolute methanol to yield concentrations of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, and 22.5 mg. per 100 cc. Similarly, from a stock solution of digitoxin in absolute methanol containing 50 mg. per 100 cc. we prepared dilutions containing 5, 10, 15, 20, 25, 30, and 40 mg. per 100 cc.

In Fig. 1 typical results are shown graphically by plotting the maximum color development in terms of the optical density against the concentration in mg. per 100 cc. Curves *A* and *C* were obtained from digitoxigenin and digitoxin, respectively, using tetraethylammonium hydroxide as the alkali. *B* and *D* are the corresponding curves when sodium hydroxide is used as the alkali. Confirming our earlier observations, it is seen that in the case of digitoxigenin also, the use of tetraethylammonium hydroxide in place of sodium hydroxide resulted in an increase of approximately 100% in the color intensity of the test. The four curves also show that for each of the two alkalies used the intensity of the color developed by digitoxigenin is approximately twice that of digitoxin. This factor also approximates the ratio of the molecular weights of digitoxin (765) and digitoxigenin (373).

The curves in Fig. 1 have been re-plotted after converting the concentrations into terms of micromoles per 100 cc. as shown in Fig. 2. Curve *A* represents the locus of the points obtained from digitoxin and digitoxigenin using tetraethylammonium hydroxide as the alkali. In a similar manner curve *B* was obtained when sodium hydroxide was used as the alkali in the reagent.

It is seen that in each curve up to a concentration of approximately 35 micromoles per 100 cc. the values for digitoxin and digitoxigenin do fall on the same curve within the limits of error of the experimental method employed. In the higher concentration range the divergence in the values for the two substances increases gradually with increase in concentration. At a concentration of 65 micromoles per 100 cc. the divergence is definitely beyond the limit of error of the method. It is also interesting to note that the values for digitoxin for each curve are consistently higher than those for the corresponding digitoxigenin concentrations. This observation can be readily explained on the basis of reaction between the sugar portion of the glycoside molecule and the reagent. From the limited data which we have previously published (8) concerning the behavior of digitoxose in aqueous solution with

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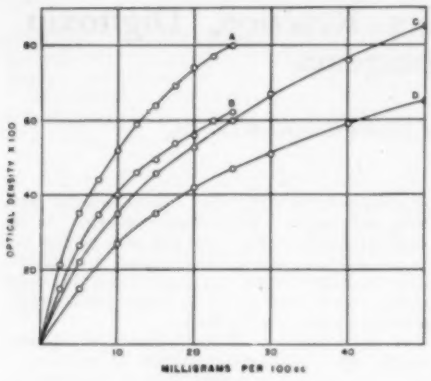

Figure 1.

A = Digitoxigenin—$(C_2H_5)_4NOH$; *B* = Digitoxigenin—NaOH; *C* = Digitoxin—$(C_2H_5)_4NOH$; *D* = Digitoxin—NaOH.

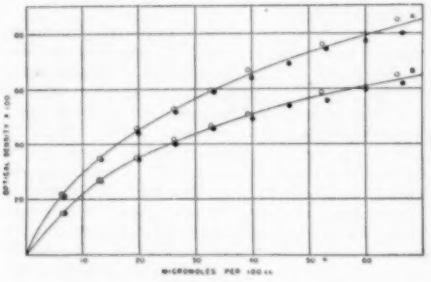

Figure 2.

A, Tetraethylammonium hydroxide as the alkali: circles = digitoxin, dots = digitoxigenin.

B, Sodium hydroxide as the alkali: circles = digitoxin, dots = digitoxigenin.

the Baljet reagent one would predict that the effect of the sugar portion of the molecule on the total color intensity would not be detectable. However, the present experiments are carried out in a strongly alcoholic medium and there is no justification in assuming that three molecules of digitoxose bound in the glycoside molecule would have the same reactivity toward the reagent as that shown in a simple aqueous solution of the sugar. Although it is clear that the sensitivity of the Baljet reaction to digitoxose is markedly less than that to the lactone structure of the glycosides, it must be remembered that the digitoxin molecule contains approximately 50% of digitoxose. Therefore in the higher concentrations of the glycoside solutions there is present the equivalent of appreciable amounts of the sugar.

In this connection it has also been suggested (9) that the reactivity of the lactone portion of the glycoside molecule is increased by the presence of the digitoxose molecules attached at C_3 of the genin. This hypothesis would appear to require that consideration be given also to the extent of possible hydrolysis of digitoxin molecule in the alkaline medium of the test solution.

CONCLUSIONS

From these observations we may draw the general conclusion that for most practical purposes the sensitivities of digitoxin and digitoxigenin on a molar basis toward the Baljet reaction are the same under the experimental conditions employed and within the concentration range examined. For more precise work and particularly in the higher concentrations a correction must be applied in the case of digitoxin to allow for the effect of the digitoxose portion of the molecule.

The significance of the behavior of digitoxigenin to the Baljet reaction with respect to our assay method for pure preparations of digitoxin is fairly obvious. Although it is doubtful that commercial preparations of pure digitoxin contain appreciable amounts of digitoxigenin, when the latter substance is present high assay values are to be expected by our method. It is generally known that the cardiotonic activity of the genin of a digitalis glycoside is usually less than that of the corresponding glycoside and this has been found to be particularly true in the case of digitoxin and digitoxigenin. Gold and his co-workers (10) have recently found that the same relationship prevails when the human assay method is employed.

If we assume that in mixtures of digitoxin and digitoxigenin the assay values obtained by both the biologic and colorimetric methods are a simple summation of the assay values for each constituent, the potency of such mixtures can be readily predicted. Thus, a sample of pure digitoxin containing 5% of digitoxigenin would probably yield a bioassay value of approximately 99%. By the colorimetric method a value of 105% would be obtained. The difference between these two values is well within the limit of reproducibility of most bioassay methods.

The data which we have presented also suggest a rather obvious and interesting speculation concerning our colorimetric assay method for digitalis preparations (11). Experience has shown that assay values obtained by that method are usually higher than those obtained by bioassay methods and frequently there are preparations which yield a much higher value by the colorimetric method. It is well known that the original glycosides in the digitalis leaf are readily broken down by enzymatic action. Does the extent or degree of such action vary with different crops of leaves and with different methods of treatment after the leaves are harvested? If we imagine the extreme case of a leaf preparation similar to the U. S. P. Reference Standard Powder in which the digitoxin is replaced by the same weight of digitoxigenin, such a product would probably yield a bioassay value of approximately 80%, while that of the colorimetric method would be 200%.

SUMMARY

The results of a quantitative study of the color reaction of digitoxigenin and digitoxin toward the Baljet reagent have been presented and discussed. It has been found that, for most practical purposes, the reactivity of the two substances on a molar basis is the same. For greater precision,

correction must be made, particularly in the higher concentrations, for the effect of the digitoxose portion of the digitoxin molecule.

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Rodent-Repellent Studies. I. Method for the Evaluation of Chemical Repellents*

By E. BELLACK and JAMES B. De WITT

A biological assay procedure and a method for the numerical expression of results have been devised for the determination of the repellency to rodents of different chemical compounds. The procedure is based upon the degree of acceptability of foods containing the candidate repellents, and has been shown to offer a rapid, reliable measure of repellent activity.

THE PRIMARY objective of the rodent-repellent studies being conducted by the Fish and Wildlife Service is the development of a treatment for paper and cardboard boxes which will prevent or minimize rodent damage to packaged goods while in storage. Such a treatment may be such that the protection is due to physical properties, such as hardness, tackiness, or grittiness, or it may be due to chemical properties, such as odor, taste, or irritancy. Examples of materials offering the first type of protection would be the relatively impervious coatings formed by waxes, plastics, or latices. Such materials might properly be termed "protectants," and will not be considered in this paper. The term "repellent," as used here, refers to those materials whose chemical characteristics are such as to cause animals to avoid contact with objects containing them.

In the initial phases of our studies it was found that little was known concerning the chemical composition of possible rodent repellents, and that it would be necessary to devise a suitable bioassay procedure permitting the rapid screening of large numbers of samples. For the purposes of simplicity of operation, the common laboratory rat was chosen as the experimental animal, and the technique described below was adopted as a standard procedure. While this technique permits the rapid screening of materials, it should be noted that it depends upon the sample coming in contact with the oral mucosa, or being ingested by the animal. Since the rat is able to gnaw through paper or cardboard without ingesting the excised particles, or even allowing them to come into contact with the soft tissues of the mouth, it is possible that a repellent material might offer little or no protection when coated upon, or impregnated in various types of packaging materials. In order to establish the ultimate utility of a repellent, it would be necessary to conduct additional tests designed to determine the most effective manner of application.

EXPERIMENTAL[1]

Experimental Animals.—The animals used in these studies were healthy, young rats, selected at random as to sex, but weighing a minimum of 150 Gm. Both albino and hooded laboratory rats were used.

Diets.—The diets used in these studies were of the type classified as a normal rat food. They were adequate for normal growth and reproduction, and were ground to pass a 10-mesh standard screen. Unless otherwise specified at the start of an experiment, the portions of treated food (i.e., food containing the candidate material) contained 2% by weight of the sample. Mixing was done by quartering and rolling, or by passing the diets through a small Wiley mill equipped with a 20-mesh screen.

* Received March 13, 1948, from the Fish and Wildlife Service, U. S. Department of the Interior, Patuxent Research Refuge, Laurel, Md.

[1] This procedure was adapted from that followed by Mr. J. F. Welch, Fish and Wildlife Service, Wildlife Research Laboratory, Denver, Colo.

Procedure.—Each experimental group consisted of three or more rats, housed in individual cages, and furnished water *ad libitum*. The rats had been maintained on the untreated experimental diets for a period of at least one week prior to the start of the test. At the beginning of the test, each animal was provided with a suitable food cup containing 20 Gm. of the untreated food, and a similar cup containing 20 Gm. of the treated food. No other food was available to the rats during the test period. The amount of food remaining in the cups was weighed each twenty-four hours during the four-day experimental period, and the cups returned to the cages. The animals, and any residual food, were discarded at the end of the experiment.[2]

DISCUSSION

One of the prime essentials in the evaluation of the results of these feeding tests was the establishment of some means for the expression of repellency. If all compounds were either completely accepted or completely rejected by the rat, the classification would be simple. However, the majority of the compounds tested fall somewhere in between these classifications, and it is desirable to be able to rate such compounds in terms of relative effectiveness. An index of repellency should permit direct comparison of the relative efficacy of materials tested at different times, and should be designed to account for a number of variables, such as the body weights of the experimental animals, the relative rates of acceptance of treated and untreated foods, the total amounts of food ingested during the test period, and the total consumption of the test material.

In the initial stages of the study, the test materials were evaluated solely on the basis of the relative acceptance of treated and untreated foods. It was assumed that any observed differences in the rates of acceptance could be attributed to the degree of repellency of the test material, and that all of the untreated food would be consumed before notable amounts of a repellent treated food would be eaten. In that case, it might be expected that all of the untreated food would be gone by the end of the second day, and that the animal then would have the choice between fasting or eating the treated food. As a final assumption, it was decided that in cases where 25% or less of the treated food was accepted during the experimental period, the test compound would be rated as a potential repellent, and would be subjected to further study.

This method of evaluating repellents was lacking in a number of respects. It did not allow for variations in the weights of the experimental animals, nor for differences in the animals' reaction to different types of compounds. Some compounds which produce physiological effects may be accepted initially, and then avoided. Other compounds may produce complete rejection of the treated food during the first two days, with slight acceptance during the remainder of the period when the hunger drive is greater. The first type might be worthless as a repellent, while the second might possess marked promise. As shown in Table I, the test scores for two such dissimilar compounds might be identical if determined on the above basis.

Although these two compounds have the same score on this basis, it is obvious that they should not be rated as equally effective repellents. The food consumption for Compound A is typical for compounds which possess no initial repellency, but produce some physiological reaction after ingestion. The figures given for Compound B are typical for materials which are truly repellent, in that the animals refused to accept any treated food until motivated by extreme hunger.

In order to arrive at a more satisfactory method for the interpretation of results, it is first necessary to define the desired pattern of repellent action. The figures given for Compound B may be taken as typical for a true repellent, in that all of the untreated food was consumed during the first two days of the test, while little or no treated food was consumed except under the drive of extreme hunger during the latter part of the experimental period. It remains, then, to develop a formula, or index of repellent action, which will account for such variables as physiological effects, random feeding, apparent attractiveness and variations in animal weights. In order to include these factors in a single index number, it is essential that the number include several parts. It should include two principal divisions, one each for the treated and untreated foods. Each division should be subdivided into four parts to represent the daily acceptance of the foods, and in order to have the expression of repellency in convenient numbers, 100 may be chosen as representing perfect repellency. The index number then would be:

$$K = 100 - (aT_1 + bT_2 + cT_3 + dT_4)(eU_1 + fU_2 + gU_3 + hU_4) \quad (1)$$

where $T_1 \ldots T_4$ represent the daily consumption of treated food; $U_1 \ldots U_4$ represent the daily consumption of untreated food, and $a \ldots h$ are weighting factors of undetermined magnitude. The product of the treated and untreated food divisions was chosen in order that the association of the two would be emphasized.

In order that the index number would reflect the desired pattern of repellent action, it was apparent that the weighting factors should be chosen so that a would have the highest value in the treated food division; b, c, and d would each be smaller than the preceding factor; while e and f must be smaller than g and h. Also, since the untreated food division should equal 1 in cases of normal acceptance, it should be divided by the total quantity of untreated food present (20 Gm.). Furthermore, since it is desired to have the index reflect the consumption in terms of milligrams per kilogram body weight, this figure should be divided by the body weight. For the purposes of simplicity of calculation, the average body weight was assumed to be 200 Gm. (0.200 Kg.) and since the values for K are relative, this will apply to any other body weight. The whole untreated food division then is divided by 100,000, since the total amount of food present (20,000 mg.) divided by the assumed body weight of the animal (0.200 Kg.) equals 100,000.

Calculation of the treated food division is more difficult. Here it is desired to express consumption

[2] Under normal conditions, rats of this weight should consume approximately 15 Gm. of food per day. Failure to ingest this amount of food may be attributed to abnormalities in the test animal, or to the effects of the candidate material in the treated food.

TABLE I.—EXAMPLES OF POSSIBLE SCORES ON THE BASIS OF FOOD CONSUMPTION

Compound	Animal Wt., Gm.	Type Bait	Daily Food Acceptance, Gm. 1 Day	2 Days	3 Days	4 Days	Score
A	150	*T*	2.0	0.0	0.0	0.0	2/20
		U	3.5	4.5	6.0	6.0	
B	275	*T*	0.0	0.0	1.0	1.0	2/20
		U	20	...	...	...	

in terms of milligrams per kilogram of the test compound. On the basis of the data from a number of tests, it was assumed that the average consumption of treated food was approximately 5 Gm., and since this food contained 2% of the test compound, this would be equal to 100 mg. of the repellent. If this value is introduced into the equation, it would be necessary to divide the weighting factors by 100 in order to bring the value of K to its former level. It is more convenient to use the original equation, and to include provision for the variation in body weights while expressing food consumption in grams:

$$K = 100 - (1/100W)(8T_1 + 4T_2 + 2T_3 + T_4)(U_1 + U_2 + 2U_3 + 4U_4) \quad (2)$$

where W is the body weight in kilograms.

Although this form of the equation proved satisfactory in a number of cases, it was found to be inadequate in cases of physiological repellency where a considerable fraction of the untreated food remained at the end of the experimental period. The low consumption of both treated and untreated food tended to give a relatively high score to compounds which could not be considered satisfactory repellents. To compensate for this, a fifth term (X), representing the amount of untreated food remaining at the end of the test period, was added to the untreated food division. In order to place proper emphasis on this term, it was assigned a weighting factor sufficiently high to produce a marked effect on the value of K. The final equation then became:

$$K = 100 - (1/100W)(8T_1 + 4T_2 + 2T_3 + T_4)(U_1 + U_2 + 2U_3 + 4U_4 + 8X) \quad (3)$$

This equation for the calculation of the degree of repellent action has been applied to the data from tests on some 1800 compounds. It has been found to furnish a rapid means for the evaluation of these data, and to allow ready comparison of the results obtained with different materials tested at different times. Examples of the results on compounds selected at random from our files are presented in Table II.

The data in Table II are typical of those obtained in the routine feeding tests. Inasmuch as relatively few animals are used in such tests, it might be questioned whether the data would be reproducible in subsequent trials. In order to determine this, and to establish the accuracy of the method, a series of tests were run on five compounds which appeared

TABLE II.—EXAMPLES OF K VALUES CALCULATED FROM EXPERIMENTAL DATA

Sample No.	Animal Weight, Kg.	Type Bait	Daily Food Consumption, Gm.[a] 1 Day	2 Days	3 Days	4 Days	K Value
701	0.335	T	0.3	0.4	2.3	1.7	94.5
		U	16.3	3.7	...	...	
702	0.268	T	0.0	0.0	1.0	0.0	98.5
		U	20	...	...	...	
703	0.237	T	0.7	0.3	1.7	0.3	86.5
		U	1.0	9.7	2.7	6.0	
704	0.185	T	0.0	0.0	2.0	0.0	95.7
		U	12.0	8.0	...	...	
705	0.191	T	0.0	0.0	0.7	0.7	97.9
		U	15.0	5.0	...	...	
706	0.165	T	0.7	0.3	2.5	1.5	78.7
		U	7.0	7.0	3.0	0.0	
707	0.186	T	0.0	0.0	0.3	0.0	99.4
		U	16.0	4.0	...	...	
708	0.155	T	0.3	5.7	2.0	3.7	59.1
		U	15.7	4.3	...	...	
709	0.167	T	2.7	3.0	1.0	4.0	−36.4
		U	7.0	4.7	2.3	1.7	
710	0.159	T	4.3	6.0	5.7	4.0	17.1
		U	11.7	5.0	3.3	...	
711	0.151	T	0.3	0.4	3.7	8.0	73.1
		U	12.3	7.7	...	...	
712	0.146	T	1.7	3.0	5.0	8.0	47.9
		U	12.3	7.0	0.7	...	
713	0.231	T	0.0	2.0	1.3	4.0	80.7
		U	11.7	4.3	0.7	1.6	
714	0.192	T	3.7	11.7	4.7	...	11.1
		U	15.0	5.0	...	...	
715	0.172	T	0.3	0.4	1.0	1.6	47.5
		U	3.7	0.6	0.7	1.7	

[a] ... indicates that all food had been consumed prior to weigh period.

to be active repellents. The resulting data are summarized in Table III.

As shown by the relatively low standard deviations from the means, the values for K are readily reproducible, and it is felt that this procedure furnishes a satisfactory procedure for the evaluation of the degrees of repellent activity shown by different chemical compounds. Through its use it has been found possible to assign numerical ratings to the 1800 compounds which have been examined, to obtain a measure of the relative efficacy of different classes of compounds and substituent groups, and to select those compounds and groups of compounds offering the greatest promise for further study.

TABLE III.—K VALUES OBTAINED IN A SERIES OF EXPERIMENTS ON SELECTED COMPOUNDS

Sample No.	No. of Observations	K Values			Std. Deviation from Mean
		Min.	Max.	Mean	
1400	24	75.0	100	86.8	8.80
1414	27	78.7	100	95.1	6.99
1427	27	80.5	100	94.5	5.04
1434	22	72.0	97.5	92.5	5.00
1469	84	85.0	100	98.01	2.78

SUMMARY

A biological assay procedure and a method for the numerical expression of results have been devised for the determination of the repellency to rodents of different chemical compounds. The procedure is based upon the degree of acceptability of foods containing the candidate repellents, and has been shown to offer a rapid, reliable measure of repellent activity.

Certain Chemical Plant Growth Regulators and Alkaloid Formation in *Datura stramonium* L.*

By D. P. N. TSAO and H. W. YOUNGKEN, JR.

***Datura stramonium* L. plants were grown in nutrient solutions to which pyridine, piperidine, atropine sulfate, and 2,4-dichlorophenoxy acetic acid were added. Plants were grown to maturity and total alkaloid formation was measured. It is concluded that these growth-regulating substances in tolerated doses do not significantly alter the biosynthesis of *Datura* alkaloids.**

IN PLANT growth treatments which make use of organic chemicals the problem arises as to what effect the growth regulator has upon the formation of alkaloidal constituents. Youngken and Fischer (1) have shown that pyridine, piperidine, and atropine sulfate will retard growth in *Datura* in large concentrations but each can be tolerated without growth effects in amounts less than 0.01 per cent (1 : 10,000). Ciamician and Ravenna (2) inoculated the above-ground portions of *Datura* and *Nicotiana* species with pyridine and piperidine and found that both substances disappeared in the plants; also that alkaloidal bases were obtained in greater yields. Pyridine produced the greatest increase. Youngken (3) described the toxic effects produced by 2,4-dichlorophenoxyacetic acid in *Datura stramonium* to show that this chemical substance is absorbed by the stem and that when so applied it had no significant effects upon total alkaloid formation in the leaves.

The extensive research of Dawson and his coworkers (4, 5, 6) clearly confirmed that the root of *Datura* and *Nicotiana* species is the sole seat of alkaloid synthesis in these plants. Thus, it would seem that organic chemical substances taken in through the underground portions would effect changes in alkaloid biosynthesis in most striking degree if at all. In view of these indications efforts were made to study the effects of pyridine, piperidine, atropine sulfate, and 2,4-dichlorophenoxyacetic acid when applied to the root and stem portions by continuous sand culture, ointment application, and inoculation methods.

EXPERIMENTAL

Normal diploid green stem *Datura stramonium* plants were grown from seed and transplanted to each of three experimental conditions, nutrient sand cultures, greenhouse soil plots, and the field. All plants selected for transplanting were as uniform in size and development as possible. Most had attained a height of from 8 to 10 inches with the first flower bud visible but unexpanded. The nutrient solution used in pure quartz sand was made accord-

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ing to a modified Hoagland formula (1). Care was taken to maintain this solution in as nearly an acid state as possible so that the more favorable factor of an alkaline medium might not influence too greatly the results. (See McNair (7).) Nutrient solutions then ranged between *p*H 6.0 and *p*H 6.37. The soil in both plots, field and greenhouse, averaged *p*H 7.13. It consisted chiefly of 1 part humus and 2 parts sand. Sand cultures for root immersion were kept saturated with 0.001% (1 : 100,000) each of pyridine, piperidine, atropine sulfate, and 2,4-D in nutrient solution by flushing with fresh solutions once a week and maintaining a constant solution table of from 2 to 3 inches. The latter was effected by adding sufficient amounts of freshly prepared solution for each treatment during interim periods of each week. These treatments were begun early in May, 1947, and continued until August 8 of the same year. The acidity and alkalinity of the solutions were observed and are reported in Table I.

TABLE I.—*p*H AVERAGES[a] DATURA STRAMONIUM HOAGLAND NUTRIENT FORMULA WITH GROWTH REGULATOR ADDED (1)

	Per Cent in N. S.	Fresh Solutions, Average *p*H	Solutions Drained Once Weekly, Average *p*H
Pyridine	0.001	6.15	7.20
Piperidine	0.001	7.10	7.42
Atropine Sulfate	0.001	5.79	6.81
2,4-D	0.001	5.18	5.83
Control	...	6.31	7.36

[a] The averages are computed from weekly determinations made during nine weeks of continuous chemical application.

Soil plots were treated with the same percentage of each chemical. Plants grown in the greenhouse soil plots were treated by applying the substance incorporated in lanolin which was then smeared on the lower stem portion approximately one inch from the base. Two such applications were made, one during the first week following transplanting and the second at the end of forty-five days of growth. Such treatments were begun in March, 1947, and roots and leaves were collected on June 5.

Attempts were made to inject the chemicals into the stem lumen of plants grown in field plots with 5 cc. of each chemical in distilled water and then 2 cc. of the same. It was found that it was possible to insert a 26-gauge needle into the green stems of young ten- to twenty-day-old seedlings and that most of the solution could be injected. Injections were attempted twice weekly. However, as each plant matured this procedure became less satisfactory and following four weeks of continuous injections the method was discontinued. No abnormal morphological growth effects were observed. Leaves and root portions were collected at once and dried for alkaloid study.

As was expected from previous reports in the literature (1) plants treated with solutions of pyridine, piperidine, and atropine 0.001%, each in sand cultures, developed as vigorously as did the controls. It was interesting to note that following six weeks of continuous growth in such chemical media *Datura* plants receiving piperidine and atropine appeared stimulated slightly and most grew larger than did the controls (Fig. 2 and Fig. 3). (Note weight factors in Tables II and III.) Plants receiving 0.001% 2,4-D, on the other hand, showed toxic effects (Fig. 1) comparable to those reported previously by Youngken (3) when he used 0.1% to 5.0% ointment and applied it to stem parts. Leaves showed marked thickening, increased pubescence, and roughening. On the other hand, stems generally remained upright. Several spineless capsules developed. It

Fig. 1.—*Datura stramonium* L. (60 days). Left: Plant treated with 0.001% 2,4-D in sand culture. Right: Untreated control.

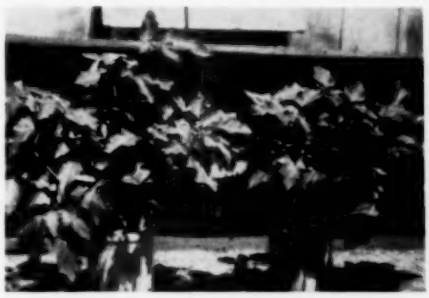

Fig. 2.—*Datura stramonium* L. (60 days). Left: Plant treated with 0.001% piperidine in sand culture. Right: Untreated control.

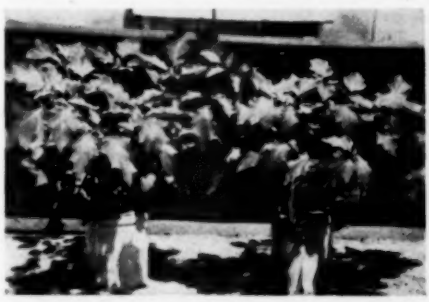

Fig. 3.—*Datura stramonium* L. (60 days). Left: Plant treated with 0.001% atropine sulfate in sand culture. Right: Untreated control.

TABLE II.—DATURA STRAMONIUM: PER CENT OF TOTAL ALKALOID FOLLOWING CHEMICAL TREATMENT IN SAND CULTURE: DRY WEIGHT CALCULATIONS

	Wt. Root Material, Gm.	Lot No.	Per Cent Total Alkaloid	Wt. Leaf Material, Gm.	Lot No.	Per Cent Total Alkaloid
Pyridine, 1:100,000	33.015	1	0.426	63.000	1	0.307
		2	0.418		2	0.329
		3	0.379		3	0.326
		Av.	0.408		Av.	0.320
		SD+[a]	0.019		SD+[a]	0.051
Piperidine, 1:100,000	42.501	1	0.381	76.400	1	0.287
		2	0.402		2	0.258
		3	0.317		3	0.289
		Av.	0.366		Av.	0.278
		SD−	0.023		SD+	0.009
Atropine Sulfate, 1:100,000	42.210	1	0.391	75.346	1	0.299
		2	0.476		2	0.334
		3	0.431		3	0.331
		Av.	0.432		Av.	0.321
		SD+	0.043		SD+	0.052
2,4-Dichlorophenoxy Acetic Acid (2,4-D), 1:100,000	31.510	1	0.424	45.012	1	0.314
		2	0.372		2	0.344
		3	0.450		3	0.336
		Av.	0.415		Av.	0.331
		SD+	0.026		SD+	0.062
Controls	46.201	1	0.467	74.760	1	0.253
		2	0.298		2	0.282
		3	0.404		3	0.271
		Av.	0.389		Av.	0.269

[a] SD = Significant Difference.

TABLE III.—DATURA STRAMONIUM: PER CENT TOTAL ALKALOIDS FOLLOWING USE OF NONTOXIC AMOUNTS 2,4-D, IN SAND CULTURE: DRY WEIGHT CALCULATIONS

	Wt. Root[a] Material, Gm.	Wt. Leaf Material, Gm.	Lot No.	Per Cent Alkaloid		
2,4-D, 0.001% (Toxic Check)	11.406	28.000	1	0.259		
			2	0.240		
			3	0.259	Av.	0.253
					SD−	0.012
2,4-D, 0.0001%	19.801	50.811	1	0.244		
			2	0.236		
			3	0.242	Av.	0.240
					SD−	0.025
2,4-D, 0.00001%	14.600	43.107	1	0.257		
			2	0.270		
			3	0.273	Av.	0.266
					SD+	0.001
Controls	13.764	42.045	1	0.263		
			2	0.265		
			3	0.268	Av.	0.265
					SD+	0.300

[a] Root material was not assayed for alkaloids because of insufficient material.

was, therefore, concluded that although 2,4-D when applied in ointment to the stem in the strength of 0.001% was nontoxic, it was absorbed by the root portions more completely in the same strength so that toxic manifestations then resulted. The leaves and roots of all plants treated during the summer of 1947 were collected, dried quickly at a temperature of 80° F., and set aside for alkaloid assay.

Due to the above toxic effects observed following the use of 2,4-D of 0.001% dilution in sand culture it was found necessary to reduce the strength of the chemical for root treatments so that an alkaloid study could be based upon a nontoxic effect as well as a toxic effect. Therefore, a fourth experiment was attempted beginning in October, 1947, during which plants were grown in cultures to which 0.001%, 0.0001%, and 0.00001% of 2,4-D in nutrient solution were added.[1] The toxic growth effects previously noted following treatments with 0.001% (1:100,000) were confirmed. Plants receiving lesser amounts of 2,4-D exhibited no toxic effects. In general these appeared stimulated and two plants

[1] Acknowledgment is made to Mr. V. Gradalin, University of Washington, who assisted with this phase of the growth study.

surpassed growth of the best controls. Following three months of growth leaves and roots were collected and made ready for alkaloid analyses (Table III).

Alkaloid Determinations.—The root and leaf materials collected from each experimental condition as described above were dried at approximately 27° C., ground into a No. 40 powder, and assayed for total alkaloids according to the U. S. P. XIII method for Stramonium (8). In cases where there was insufficient material available the standard procedure was modified so that smaller weight samples could be analyzed. The findings of these assays are presented in Tables II, III, IV, and V.

sulfate, and 2,4-D. The fact that the first two heterocyclic compounds are structurally similar to portions of the hyoscyamine molecule yet do not take part significantly in its biosynthesis suggests that the *Datura* alkaloids are quite stable within the plant and might not be directly correlated with "precursor" molecules of such a nature. It appears also that none of these substances nor their conversion products were utilized to any large degree as nitrogen sources for alkaloid synthesis.

The evidence indicating that each substance was absorbed by root and leaf portions of the plant is largely based upon morphological growth changes that can be observed following toxic doses. None

TABLE IV.—DATURA STRAMONIUM: PER CENT TOTAL ALKALOIDS FOLLOWING OINTMENT APPLICATIONS TO STEM BASES: GROWTH IN SOIL PLOTS

	Wt. Root Material, Gm.	Lot No.	Per Cent Total Alkaloids	Material, Gm.	Lot No.	Per Cent Total Alkaloids
Pyridine, 1:100,000	40.1	1	0.363	69.1	1	0.355
		2	0.442		2	0.357
		3	0.420		3	0.367
		Av.	0.408		Av.	0.359
		SD	0.015		SD+	0.016
Piperidine, 1:100,000	48.3	1	0.491	73.0	1	0.337
		2	0.570		2	0.428
		3	0.483		3	0.393
		Av.	0.515		Av.	0.386
		SD+	0.122		SD+	0.043
Atropine Sulfate, 1:100,000	45.7	1	0.343	76.2	1	0.218
		2	0.388		2	0.315
		3	0.412		3	0.214
		Av.	0.381		Av.	0.249
		SD−	0.012		SD−	0.094
2,4-D, 1:100,000	44.6	1	0.311	70.4	1	0.305
		2	0.461		2	0.273
		3	0.377		3	0.319
		Av.	0.383		Av.	0.299
		SD−	0.010		SD−	0.044
Controls	48.1	1	0.380	73.1	1	0.264
		2	0.392		2	0.421
		3	0.406		3	0.343
		Av.	0.393		Av.	0.343
		SDI	0.300		SDI	0.300

From the data obtained it is evident that plants treated with piperidine and atropine sulfate grew most favorably. Treatment with pyridine and 2,4-D, on the other hand, resulted in lower amounts of dried root and leaf material for assay. Yet the total alkaloid yields obtained from this less favorable growth were not always lower than those obtained from better plants. Therefore, there seems to be little correlation between the nature of the plant growth and its ability to form alkaloids. For example, plants receiving piperidine in sand culture grew better than those receiving 2,4-D. The latter plants, however, yielded averagely more alkaloid.

DISCUSSION

The above findings indicate in general that there is no significant effect produced on alkaloid synthesis in *Datura stramonium* when the root and above-ground portions are exposed for long growth periods to nontoxic quantities of pyridine, piperidine, atropine

of the trace dilutions could satisfactorily be tested for in so minute a quantity.

The fact that neither toxic nor nontoxic quantities of 2,4-dichlorophenoxyacetic acid effect any appreciable change in total alkaloid yield further suggests the probability that neither this substance nor its conversion products have any appreciable precursor value in *Datura* alkaloid synthesis. This confirms the findings of Youngken (3) who earlier showed that no significant alkaloid change was effected when stems were treated with 2,4-D ointment. It might imply also that alkaloid synthesis in *Datura* is not associated with somatic cell growth changes for it was definite that toxic quantities of 2,4-D did alter cell growth markedly but at the same time had no unusual effect upon alkaloid formation. Unless shown otherwise by the use of other organic substances it would seem, then, that alkaloid synthesis in *Datura* is a mechanism more closely allied with genetical factors (9) and that while it can be influenced by nitrogen-supplying elements, especially late during the growth period (10), these must be of simple structure.

TABLE V.—DATURA STRAMONIUM: PER CENT TOTAL ALKALOIDS FOLLOWING INJECTIONS OF CHEMICALS INTO MEDULLA OF STEMS: GROWTH IN SOIL PLOTS

	Wt. Leaf Portions, Gm.	Lot No.	Per Cent Total Alkaloids		
Pyridine, 1:100,000	104	1	0.307		
		2	0.298		
		3	0.342		
		4	0.323	Av.	0.317
				SD−	0.077
Piperidine, 1:100,000	117	1	0.371		
		2	0.264		
		3	0.411		
		4	0.376	Av.	0.355
				SD−	0.039
Atropine Sulfate, 1:100,000	107	1	0.305		
		2	0.311		
		3	0.346		
		4	0.327	Av.	0.322
				SD−	0.072
2 4-D, 1:100,000	106	1	0.283		
		2	0.301		
		3	0.276		
		4	0.354	Av.	0.303
				SD−	0.091
Controls	110	1	0.311		
		2	0.425		
		3	0.379		
		4	0.361	Av.	0.394
				SD±	0.300

SUMMARY

A study of the effects of certain plant growth regulators on total alkaloid yields in roots and leaves of *Datura stramonium* has been made.

Contrary to the report of Ciamician, *et al.*, there appears to be no significant effect on the yield of total alkaloids following continuous applications of pyridine and piperidine. Further, it has been shown that atropine sulfate does not significantly influence total alkaloid yields when induced into the plant by several methods.

Finally, a similar study employing toxic and nontoxic quantities of 2,4-D has confirmed the findings previously reported by Youngken (3) and has shown that during toxic growth in the plant caused by this substance there is no significant altering of total alkaloid in the leaves.

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Polarographic Studies of Diethylstilbestrol*

L. E. BINGENHEIMER, JR., and JOHN E. CHRISTIAN

Studies on the polarographic properties of diethylstilbestrol were carried out, with the idea of developing a polarographic assay for this and other synthetic estrogens. Polarographic reduction of diethylstilbestrol was not found to occur under the conditions studied. Minute quantities of the estrogen suppress the oxygen maximum in a solution of potassium chloride, the degree of suppression being approximately proportional to the concentration of diethylstilbestrol within a narrow range. This property is the basis for a suggested assay of preparations of diethylstilbestrol.

ALTHOUGH the polarographic properties of the natural estrogens have been thoroughly investigated, little appears to have been done with the synthetic estrogens. Present assays of synthetic estrogens fall almost entirely into the classes of colorimetric, spectrophotometric, and biological analysis. Wessely and Wratil (1) have taken a step in the direction of polarographic analysis of stilbestrol and related compounds, but without encouraging results. The authors have undertaken a series of studies to devise a workable polarographic analysis applicable to this group of compounds.

EXPERIMENTAL

The instrument used in these experiments was the Sargent Model XX visible recording polarograph. An attempt was made to maintain constant conditions throughout the entire work. A column of 40 cm. of mercury was maintained in the standpipe of the dropping electrode. All solutions were allowed to come to room temperature, which was maintained within a range of 25 ± 1°. For the quantitative part of the work, the following control settings were used: sensitivity, 3; sensitivity vernier, 25; initial potential, 0; span potential, 3; damping, 3.

In the recording of oxygen maxima, which generally occurred at a potential of not over −0.6 volt, multiple determinations were recorded on a single chart by returning the bridge control to zero following the drop in current after each maximum.

The first experiments were to determine what type, if any, of polarographic behavior is exhibited by stilbestrol. Electrolysis of solutions containing stilbestrol in concentrations from 10^{-4} to 3×10^{-4} molar failed to give polarographic reduction waves when the supporting electrolyte was 0.1 *N* potassium chloride in approximately 30% aqueous ethanol, and again when the supporting electrolyte was potassium chloride in 0.1 *N* potassium hydroxide. No polarographic oxidation was observed when the same solutions were electrolyzed with polarity of the electrodes reversed.

Stilbestrol completely suppressed the oxygen maximum in 0.001 *N* solutions of potassium chloride in 10% aqueous ethanol and in 0.01 *N* potassium hydroxide. The latter solution appears to be more desirable, as ethanol has a slight suppressing action which interferes with the measurement of the activity of the stilbestrol.

For purposes of measurement, bubbling of oxygen, rather than air, through the solution was found to give much larger, more satisfactory maxima. These maxima were completely suppressed by as little as 10^{-6} molar stilbestrol, but almost no suppression was observed when the concentration was reduced to 10^{-6} molar.

As a basis for quantitative work the concentration of stilbestrol necessary to suppress the oxygen maximum by 50% was desired. This procedure was applied to alkaloids by Stackelberg and Schutz (2). A range of solutions from 0 to 10^{-5} molar in potassium chloride-potassium hydroxide supporting electrolyte were saturated by bubbling with oxygen for ten minutes. In all cases the degree of maximum suppression was approximately proportional to stilbestrol concentration from 0.2×10^{-5} to 0.8×10^{-5} molar, with approximately 0.4×10^{-5} molar required for 50% suppression.

Repeated electrolysis of solutions resulted in progressive decrease in the size of the maxima. This decrease is apparently irreversible. Further treatment of the solutions with oxygen did not restore the size of the maxima. A lowering of the *p*H of solutions after electrolysis was noted, but this effect probably is unrelated to the height of the maximum. When a supporting electrolyte corresponding to the buffer of Britton and Welford (3), containing potassium acid phosphate and sodium hydroxide (*p*H 11.45 at 25°), was used the tendency of the maxima to decrease in size was unchanged. The buffer exhibited a degree of maximum suppression which interfered with the measurement of stilbestrol.

Statistical evaluation of the results of repeated determinations of the same solutions showed that the per cent mean deviation in maximum size increases as the degree of suppression increases.

Tablets of stilbestrol were assayed polarographically by the maximum-suppression method. Extraction of the tablets with ether and sodium hydroxide test solution was carried out as described in the U. S. P. XIII assay for stilbestrol tablets, with the exception that an added extraction step through ether and base was found necessary to remove gelatin which interfered with the results. The residue after final evaporation of the ether was taken up in sodium hydroxide test solution. Potassium chloride was added and the solution diluted to correspond to 0.4×10^{-5} molar stilbestrol in 0.01 *N* potassium chloride and 0.001 *N* potassium hydroxide. Standards corresponding to the official limits of 90% and 110% of labeled strength were made up in a similar manner. When the solutions

* Received Sept. 27, 1948, from the School of Pharmacy, Purdue University, Lafayette, Ind.

were saturated with oxygen and electrolyzed, the size of the maximum in the unknown solution was between those of the standard solutions, indicating stilbestrol content within the desired range.

SUMMARY AND CONCLUSIONS

Diethylstilbestrol was found not to be polarographically reducible or oxidizable. It does, however, suppress the oxygen maximum, and this characteristic can be used for quantitative analysis. The degree of maximum suppression is roughly proportional to stilbestrol concentration in the range from 0.2×10^{-5} to 0.8×10^{-5} molar, with about 0.4×10^{-5} molar concentration required for 50% suppression of the maximum.

Repeated electrolysis of solutions results in decrease in maximum size, which is not restored on further treatment of the solutions with oxygen. Buffering does not prevent this action.

Quantitative determination of stilbestrol in pharmaceutical preparations by the degree of suppression of the oxygen maximum is possible. The main advantage of this method over the official assay is the time saving of about one hour. The main disadvantage of the method, especially in the assay of tablets, is the difficulty of removing gelatin, which interferes with the determination.

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Book Reviews

The Therapeutic Value of Penicillin. By DONALD G. ANDERSON and CHESTER S. KEEFER. J. W. Edwards, Ann Arbor, Mich., 1948. xiv + 297 pp. 15 x 24 cm. Price $7.00.

The Therapeutic Value of Streptomycin. By CHESTER S. KEEFER and WILLIAM L. HEWITT. J. W. Edwards, Ann Arbor, Mich., 1948. xiii + 289 pp. 15 x 24 cm. Price $7.00.

Long before penicillin and streptomycin were freely distributed, much was known about the clinical use of these drugs because of the study carried out by the Committee on Chemotherapeutics and other agencies of the National Research Council. These two reports are the summary and statistical findings of these committees; 10,000 cases in the instance of penicillin and 3000 in the instance of streptomycin are represented.

In each book, injections are broken down into convenient categories and the data are given in both tabular and discussion form. In addition, adequate attention is given to toxic and side reactions.

By using a mechanical sorting machine and by the tabulation of data on punch cards, the committee has been able to give details on apparent optimal dosage regimens, sex differences, degree of response with different complications and many other facts that would take years to accumulate in the usual manner. The amount of information to be found in these volumes of less than 300 pages is astounding.

The application of the contents of these books in a critical manner will prevent the abuse of two of the most valuable drugs the world has ever known. Nevertheless, precaution should be taken to avoid "freezing "progress under the assumption that these findings are entirely conclusive.

Bacterial and Mycotic Infections of Man. Edited by RENÉ J. DUBOS. J. B. Lippincott Co., Philadelphia, 1948. xiii + 785 pp. 17.5 x 25.5 cm. 101 illus. Price $5.00.

Under the influence of important changes in the collateral sciences of biochemistry, biophysics, physiology and pathology, bacteriology has been changing in its outlook during the past several years. A comparison of this book with past text and reference books in this field illustrates the change very forcefully. The reader will be impressed by the small amount of space devoted to morphology.

This book was written by a group of experts such as H. E. Alexander, B. D. Davis, H. Eagle, C. A. Janeway, H. R. Morgan, W. J. Nungester, A. B. Sabin, and A. M. Pappenheimer, Jr. Under the able editorship of René J. Dubos, a remarkable uniformity of style is present and there is no serious duplication of facts.

Chapters will be found on morphology, physiology, host-parasite relationships, the allergic state, immunology, blood groups, and the various bacteria and other microorganisms causing specific diseases. In addition there are chapters on principles of sterilization, principles of chemotherapy, and the cultivation and identification of pathogenic bacteria.

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